ADAPTATION OF <u>TRYPANOSOMA</u> <u>(NANNOMONAS)</u> <u>CONGOLENSE</u> BRODEN 1904 TYPES TO DIFFERENT HOSTS AND TRANSMISSION BY <u>GLOSSINA</u> SPECIES.

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APPROVAL

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ABSTRACT

The influence of host blood on midgut infection, maturation rates in *Glossina* species, transmission and virulence to mice of *Trypanosoma congolense* DNA types was investigated. The study was also conducted to identify an optimum technique for isolating *Nannomonas* stocks from the field. *Trypanosoma congolense* parasites used in this study were identified by the Polymerase Chain Reaction Technique.

Zebra and goat blood supported higher midgut and labral infection rates of *T. congolense* in *Glossina morsitans centralis* than pig, donkey, black rhinoceros, Thomson's gazelle and eland blood. Of these hosts, zebra supported the highest metacyclic loads in the hypopharynx. The proportion of midgut infections maturing (transmission index) was generally high for most blood types. It ranged from about 58.0% for tsetse infected with eland blood to 97.0% for tsetse infected with zebra blood. From a practical view point, this study suggests that areas with many goats should have higher infection rates of some *T. congolense* genotypes. Zebra being a less favoured host probably has a lesser impact on the infection rates.

The pattern of *T. congolense* Savannah, Kilifi and Riverine / Forest infection rates in *G. m. centralis* infected with goat, black rhinoceros and Boran cow blood was similar during the first 10 days of infection. Rates were highest (up to 90%) on day 3, and dropped by day 5 to reach constant values (ranging from 5% to 45%) by day 10. The 10th day midgut infection rates did not differ significantly from those on day 21 in flies infected with pig, zebra, goat and eland blood. Carbohydrate binding proteins (lectins) and other factors are considered to play a role in controlling trypanosome establishment and maturation. Male *G. m. centralis* infected with zebra and eland blood matured significantly

more midgut infections than females. A higher proportion of mice became infected with *T. congolense* when the infection was initiated by the bite of male tsetse (56.0%) than when the infection was initiated by the bite of a female tsetse (33.0%). This finding was interpreted to suggest an important impact on the application of the Sterile Male Technique (SMT) for controlling tsetse flies.

Kilifi *T. congolense* took a significantly shorter time to infect mice (13 days) than the Savannah DNA type (18 days). The Kilifi type of *T. congolense* (K60/1) killed a higher proportion of mice than the Savannah type within 45 days after infection. It also showed earlier parasitaemic peaks than the Savannah type, thus suggesting that the former (Kilifi *T. congolense*) used in this investigation is more virulent than the latter (Savannah *T. congolense*). Studies on parasite diversity have implications on trypanosomiasis control because they can provide clues to questions on factors that maintain trypanosomes at endemic or factors that can encourage trypanosomes to go into epidemic levels.

Trypanosoma congolense (Kilifi type) was detected earlier in mice when the transmitting tsetse was fed on infective goat blood (mean = 11 days) than when they were fed on infective eland blood (mean = 14 days). Transmission rates of Kilifi and Savannah types of *T. congolense* to mice were similar and were not influenced by the host blood type used to infect the transmitting fly. Therefore, the study suggests that host blood can exert host-specific effects on metacyclic development in the tsetse fly that can be subsequently observed when the animal are transmitted with the trypanosomes.

Isolation and cloning of trypanosomes by mouse inoculation were more effective in Balb/c than Swiss mice. Isolation of *T. congolense* through gut-form transfer from one tsetse species to another (procyclic expansion technique) was most effective in laboratory-

reared *G. m. centralis* and not effective in *G. pallidipes* and *G. m. morsitans*. Tsetse passaged with procyclics suspended in fresh goat blood generally survived well during the observation period (survival rates ranged from 58% to 95%). Cholesterol and D(+)-glucosamine added to the goat infective bloodmeal increased infection rates of some but not all stocks of procyclics. Double cholesterol feeds supported significantly higher midgut infection rates than single cholesterol feeds. Tsetse fed on cholesterol-trypanosome-blood mixtures survived well (survival rates ranged from about 60% to 80%). D(+)-glucosamine facilitated infection rates of procyclics in *G. m. centralis* but also caused mortality. Pocyclics incubated in different blood mixtures, including cow and goat serum, reconstituted goat red blood cells, heated or unheated, retained viability for a day at room temperature (25°C.). This suggested that the media used for cryopreserving procyclics was not a critical factor. This study suggests that it is feasible to isolate *T. congolense* types of trypanosomes by feeding laboratory - reared *G. m. centralis* tsetse on fresh goat blood and chemical additives such as cholesterol, but maintaining the flies on a rabbit. This finding is discussed in relation to other methods such as the use of *G. m. morsitans* fed on synthestic diets or the combined use of culture and laboratory reared tsetse flies to isolate *T. congolense* from wild hosts.

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CHAPTER 1

GENERAL INTRODUCTION

African trypanosomes are protozoan parasites transmitted from one mammalian host to another, mainly through the bite of tsetse flies (*Glossina* species). Trypanosomes cause a disease in humans called sleeping sickness as well as several diseases in livestock collectively referred to as 'nagana'.

Trypanosoma brucei rhodesiense and *T. brucei gambiense* cause sleeping sickness which is characterised by parasitaemia in the initial stage and central nervous disorders at a later stage (Hoare, 1970; WHO, 1993; Kuzoe, 1993). A more diverse group of parasites, *T. brucei brucei, T. congolense, T. vivax, T. simiae, T. suis, T. godfreyi* and *T. evansi* cause disease in livestock such as cattle, sheep, goats, pigs, camels, horses and donkeys. *Trypanosoma equiperdum and T. evansi* also cause disease but are found outside the 'tsetse belt'. *Trypanosoma equiperdum* is transmitted sexually among equines (mainly horses) and *T. evansi* mechanically by biting flies. *Trypanosoma evansi* is often fatal in camels (Stephen, 1986; Hoare, 1970).

A detailed assessment of the economic impact of the African trypanosomiases has not been undertaken recently because of limited reliable data on animal and vector populations. Nonetheless, general estimates indicate that animal trypanosomiasis contributes significantly to cattle mortality and losses in milk and meat production (ILRAD Report, 1991). Annual losses in meat production have been estimated at about US $ 5 billion (ILRAD Report, 1990). Maintenance of high-grade, exotic breeds of livestock in tsetse endemic areas requires the regular use of trypanocides for prevention and treatment of trypanosomiasis. Other economic losses occur indirectly through a marked reduction in animal vitality resulting in reduced work output (ILRAD Report, 1982). Furthermore, trypanosomiasis often occurs in potentially productive areas, reducing full land utilization (Jordan, 1978).

In addition to economic importance, African trypanosomiasis also contributes to human morbidity and mortality. Serious epidemics due to the Gambian and the Rhodesian forms of sleeping sickness have occurred in East and Central Africa, causing over 100,000 deaths in Uganda between 1905 and 1910 (Ford, 1971). Buyst (1974) and Rickman (1974) also recorded sleeping sickness outbreaks in the Luangwa Valley of Zambia. Although massive epidemics are no longer reported, the disease continues to afflict people in endemic areas. At the Uganda / Sudan border, for example, 2,208 people suffering from sleeping sickness were treated in 1990 alone (WHO, 1993). Drugs in use for treating sleeping sickness include pentamidine for *T. gambiense* (early-stage), suramin for *T. rhodesiense* (early-stage) and melarsoprol for the late stage. The arsenical drug, melarsoprol, has been associated with side-effects which can be fatal (Hajduk *et al.*, 1992). Recently, a new drug, eflornithine, has been introduced for treatment. It is safer and tolerated better by patients, but it is expensive; being estimated at about US $ 500 per patient (Kuzoe, 1993).

Several methods have been used to control tsetse flies. These include destruction of forests, clearing of bushes inhabited by tsetse and use of residual and non-residual insecticides alone or in combination with traps and targets. According to Jordan (1974) each method has some drawbacks. Bush clearing and felling of trees over large areas for example, control tsetse but the strategy is environmentally unacceptable since it destroys important natural resources. Use of insecticides with traps and / or targets has been successful (Jordan, 1978, 1974; Vale *et al.*, 1986), but the method is not popular due to its detrimental effects on the environment, especially when appplied over large areas. Traps and targets have been demonstrated to reduce fly populations but only in some types of areas and for some tsetse species (Vale *et al.*, 1986). Furthermore, traps and targets require regular servicing and good co-operation of local communities in areas where the control measures are being undertaken (ILRAD Report, 1990).

Some biological features of the trypanosomes also hinder trypanosomiasis control. African trypanosomes, for example, are capable of changing the composition of their exposed antigens. This antigenic variation presents the host with a large number of surface antigens which allows a proportion of the trypanosome population in the bloodstream to survive, despite the immune response of the host. Indeed, the failure to produce an effective vaccine against trypanosomes has been attributed to the large numbers of surface antigens (Vickerman, 1978).

Effective control of trypanosomes also requires accurate detection and identification of the organisms in order to assess the extent of their distribution in both the insect vector and the animal host. Highly sensitive and specific biochemical methods based on the structure of DNA and enzyme polymorphism are being used for detection or identification of trypanosomes from tsetse and mammals (McNamara *et al.*, 1989; Young and Godfrey, 1983). Use of these methods has revealed a large diversity of genotypes within *T. congolense* which has led to the designation of the following three main groups: Savannah, Riverine/Forest and Kenya Coast, corresponding to ecological and geographical zones of Africa (Gashumba, 1990). At present, the distribution of these *T. congolense* genotypes in Africa is not well understood. Similarly, their biological characteristics such as responses to trypanocides and infectivity to livestock, wildlife and tsetse flies remain under speculation. The inherent difficulty in the field of isolating *T. congolense* from tsetse and animal hosts has also complicated epidemiological studies of these organisms (Dukes *et al.*, 1991). The role of various species of wildlife in maintaining these types (strains) of *T. congolense* is unknown. Studies on effects of host blood on the development of *T. congolense* in different hosts are therefore desirable as they reveal useful information regarding these unknown factors.

General objectives

The broad objective of the present study is to provide information on the biological characteristics of *T. congolense* types in tsetse flies and laboratory animals and suggest possible application of such information in controlling such parasites.

Specific objectives

Specific objectives of this study were to:

(1) isolate, clone and identify *T. congolense* types from the field.

(2) compare infectivity of selected *T. congolense* types to *Glossina* species when fed on different host blood types.

(3) compare maturation and cyclical transmission rates of *T. congolense* types from tsetse flies into mice.

(4) compare virulence of *T. congolense* types to mice.

Definitions

    i. Isolate: A section of a population from a naturally infected host introduced into artificial conditions of maintenance, usually by inoculation into animals or into cultures (Lumsden, 1971; Turner, 1990).

    ii. Stock: A population derived from an isolate (Turner, 1990).

    iii. Stabilate: A population whose reproduction has been 'arrested' by viable preservation (Lumsden, 1971).

    iv. Clone: A population derived from a single organism (Murray *et al.*, 1983).

    iv. Infectivity: Is a measure of the ability of a disease agent to establish itself in a host (Jawetz *et al.*, 1980).

    v. Virulence: The severity of a disease, often quantified as LD_{50} i.e. the individual dose or numbers of parasites which will kill 50% of susceptible animals under controlled conditions (Jawetz *et al.*, 1980).

CHAPTER 2

LITERATURE REVIEW

2.1 Systematics.

A. Tsetse flies.

Tsetse flies belong to the genus *Glossina* within the family Glossinidae (Jordan, 1986). Based on the morphology of male and female genitalia, the genus has been subdivided into three groups as follows: *morsitans, fusca* and *palpalis* (Potts, 1970, 1973). This taxonomic grouping is supported by habitat preferences and genetic studies (Jordan, 1974, 1986; Carlson *et al.*, 1993). Systematics of tsetse flies have been studied in details and are well documented (Potts, 1970). To-date more than 22 species and sub-species have been identified and described (Jordan (1986).

B. Trypanosomes

Trypanosomes belong to the protozoan genus *Trypanosoma* of the family Trypanosomatidae. They infect various vertebrate animals ranging from amphibians (e.g. frogs) to mammals (Myler, 1993). The genus is sub-divided into several sub-genera, species and sub-species (Hoare, 1970, 1972; Molyneux and Ashford, 1983), but the placing of organisms in some of these taxonomic ranks is still under debate (Myler, 1993). This is particularly true for organisms within the *Trypanozoon* and *Nannomonas* subgenera. *Trypanosoma congolense, T. simiae* and *T. godfreyi* are the three recognised species within the subgenus *Nannomonas* (Hoare, 1970; McNamara *et al.*, 1994). The taxonomic history of these trypanosomes has been controversial due to their large morphological diversity. This has led, for example, to several synonyms of *T. congolense*

and *T. simiae* (Hoare, 1972). This taxonomic 'confusion' has important implications on the proper understanding of the biology of trypanosomes needed for disease control.

2.2 Distribution and vectorial ability of *Glossina m. centralis.*

The distribution maps of *Glossina* were revised by Katondo (1984), Jordan (1986) and Moloo (1993a). *Glossina m. centralis* is one of the *morsitans* groups of tsetse flies which are the most widely distributed, extending from East Africa to West Africa, through Central Africa (Katondo, 1984; Jordan, 1986) (Fig. 1).

Glossina m. centralis is susceptible to various trypanosome species, including *T. congolense, T. vivax* and *T. b. brucei* (Moloo *et al.,* 1992a; Moloo *et al.,* 1994a; Mihok *et al.,* 1994a). Under laboratory conditions *G. m. centralis* has been shown to have a higher vectorial capacity for trypanosomes than the *palpalis* and *fusca* groups, as well as other morsitans groups such as *G. pallidipes* and *G. m. morsitans* (Shaw and Moloo, 1991; Ndegwa *et al.,* 1992;. Moloo *et al.,* 1992a; Mihok *et al.,* 1994a; Mihok *et al.,* 1995). Mihok *et al.* (1994a) recently demonstrated cyclical development of gut-form trypanosomes transferred into *G. m. centralis* from donor tsetse. This observation suggests potential use of this fly species in isolating trypanosomes.
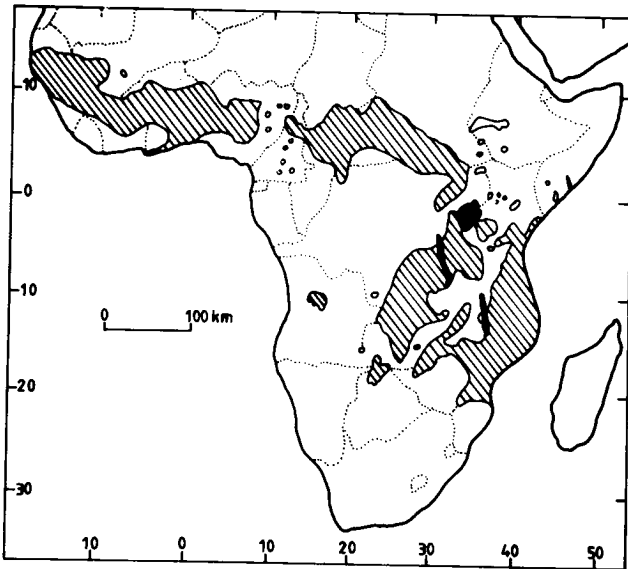
Figure 1 Distribution of the *morsitans* group tsetse in Africa. Modified from Jordan (1986).

2.3 Host-parasite interactions.

A. Host-related factors.

Knowledge of the role played by hosts (wildlife and livestock) in the epidemiology of trypanosomiasis is vital for effective disease control. Many crude surveys carried out to determine trypanosome infection rates in wildlife species in the past revealed general information on the prevalence of trypanosome species (Dillman and Townsend, 1979; Keymer, 1969). However, the specific mechanisms responsible for disease transmission cycles between and among wildlife and livestock species remained poorly understood, due to small population sizes used in most cases (Wells and Lumsden, 1968). The problem has been compounded by the difficulty of isolating wildlife strains of trypanosomes. This is due to various reasons, including: (1) the lack or limited availability of specialised expertise for collecting blood samples from wildlife, (2) the failure of many *Nannomonas* trypanosomes to grow in laboratory animals, using the available parasite isolation techniques and (3) low parasitaemias that occur in some of these wild species (Dillman and Townsend, 1979; Dukes *et al.*, 1991).

Ashcroft *et al.* (1959) revealed wide variations in susceptibility of various wild species to trypanosomes when the animals were experimentally infected with *T. b. rhodesiense* and *T. b. brucei* under controlled conditions. Based on these differences, game animals were divided into two groups, namely, group one which consisted of host species that died of infection, including monkeys and Thomson's gazelle, and group two which consisted of animals that resisted or 'tolerated' the infections, including baboons, bush-pigs and Wart hogs. Experimental infections of a limited number of game animals with *T. congolense* revealed that most of the animals did not succumb to the infection or their parasitaemia was of short duration and the animals recovered. Subsequently, resistance to trypanosome infection by wildlife and livestock species has been reported by several researchers (Rickman and Kolala, 1982; Grootenhius *et al.*, 1990; Olubayo *et al.*,

1991; Mihok *et al.*, 1991). Rickman and Kolala (1982) reported that sera from eland, waterbuck and to a lesser extent, spotted hyaena and hippopotamus, kills bloodstream forms of trypanosomes *in vitro* and destroys trypanosome infectivity to laboratory rodents. Olubayo *et al.* (1991) compared the ability of a wildlife species (waterbuck) and a domestic animal (Boran cow) to resist trypanosome infection by exposing the animals to *G. morsitans* infected with *T. congolense*. The appearance of parasites in peripheral bloodstream was delayed in the waterbuck, and the levels of intermittent parasitaemia and anaemia were much lower in this animal than in the cow. Several investigations have provided information on how wildlife and livestock species naturally control trypanosome infections (ILRAD, 1982; Mulla and Rickman, 1988a, b; Murray and Dexter, 1988; Olubayo *et al.*, 1990). Despite this advance in knowledge, information on host effects on maturation and transmission rates of trypanosomes in the tsetse vector is scanty.

The type of animal on which tsetse flies feed is considered an important factor that determines trypanosome infection rates of the insects. Jordan (1965) demonstrated a close relationship between total infection rates in *Glossina* spp. and the proportion of bloodmeals obtained from bovids. This was done by relating data on animal food sources (Weitz, 1963) with that on trypanosome infections collected in the same localities of Nigeria. Close correlation between *T. vivax* infection rates in tsetse and proportion of feeds on bovids has been well established (Moloo *et al.*, 1980; Snow *et al.*, 1988). Effects of host blood on trypanosome infection in tsetse vectors were investigated by Moloo (1981) who infected groups of *G. m. morsitans* with *T. congolense, T. brucei* and *T. vivax,* and maintained them on several hosts including cows, rabbits and mice. The author observed that flies maintained on rabbits and cows had the highest tsetse infection rates with *T. brucei* and *T. vivax* while those fed on mice had the lowest infection rates with the same trypanosome species. In the case of *T. congolense* infections, however, significantly higher infection rates were obtained when the flies were fed on goats and rabbits as maintenance hosts. These observations were thus interpreted to suggest that the type of

animal species on which *G. m. morsitans* are maintained after an infective feed influences subsequent infection rates in the vector. Similarly, Mihok *et al.* (1993) investigated the effects of blood from selected domestic and wildlife species on infection rates in *G. m. morsitans* and *G. m. centralis.* In so doing blood was fed to tsetse flies through a silicone membrane and the insects were maintained on a rabbit. Goat blood produced the highest trypanosome infection rates in the flies while eland produced the lowest. This finding stimulated other questions, such as whether the facilitation effect was unique to one breed of goats or applied to other breeds as well; influence of goat blood on maturation rates of trypanosomes, especially, the newly discovered *T. congolense* DNA types and the influence of such blood on transmission of trypanosomes to other hosts.

B. Tsetse-related factors

Maudlin (1982) and later, Maudlin and Dukes (1985) showed that lines of *G. m. morsitans* highly susceptible or refractory to *T. congolense* and *T. b. brucei* could be selected in the laboratory. These observations suggested that susceptibility or refractoriness to trypanosome infections may be under genetic control in the fly. Ibrahim *et al.* (1984) showed that extracts of midgut and hindgut of *G. austeni* could agglutinate *T. b. brucei* procyclic forms. Although it was known that insects could produce agglutinins to parasites *in vitro*, their role *in vivo* remained speculative. However, Maudlin and Welburn (1987) demonstrated *in vivo* that by specifically blocking tsetse midgut lectins using amino sugars, they could significantly increase infection rates of trypanosomes that have a midgut stage in tsetse flies. They fed *G. m. morsitans* on blood containing *T. congolense* or *T. b. brucei* on day one and D(+)-glucosamine for five days and dissected the flies 21 days post-infection. Addition of D(+)-glucosamine produced significantly higher midgut infections than in control flies fed on D-galactose. It was concluded that D(+)-glucosamine given during the first day of the infection strongly inhibited the killing

of *T. congolense* and *T. brucei brucei* in tsetse midgut. Subsequently, Welburn *et al.* (1989) showed that the rate of midgut killing varied within and between tsetse fly species. Stiles *et al.* (1990) reported increased agglutination and lysis activities towards *T. congolense* and *T. b. brucei* by the posterior midgut of *G. palpalis* after a bloodmeal, but low activities in starved flies. Low levels of agglutinin properties during tsetse starvation correlated with increased midgut infection. Therefore, increased susceptibility to trypanosome infections observed in starved tsetse was attributed to low lectin levels during this period. It was proposed that the presence of bacterial symbionts referred to as Rickettsia-Like-Organisms (RLOs) in tsetse midgut cells might be involved in lectin production (Maudlin and Ellis, 1985). Since RLOs were found in the ovaries of female tsetse flies, Maudlin (1991) considered that these bacterial symbionts are transovarially transmitted from adult tsetse flies to offspring. Maudlin (1991) suggested that the critical events influencing susceptibility of young flies to trypanosome infection were taking place in the larval-pupal stage. They proposed that D(+)-glucosamine accumulates during this time due to chitinase from RLOs hydrolysing chitin from the peritrophic membrane. This build-up of D(+)-glucosamine due to chitinase activity was thought to be responsible for the increased susceptibility to trypanosome infection in teneral flies. Moloo and Shaw (1989) argued that newly emerged (teneral) flies which were known to be more susceptible to trypanosome infection (Wijers, 1958) would possess more rickettsia organisms than old (non-teneral) flies if the presence of RLOs in tsetse midgut was positively correlated with trypanosome infection in tsetse flies. However, their studies, using electron microscope, revealed the opposite result. RLOs were found to be more numerous in the non-teneral than in teneral tsetse flies. These bacterial symbionts were also found in all the experimental tsetse flies regardless of whether the flies were susceptible to trypanosomes or not. It was therefore concluded that the ability of trypanosomes to establish and develop in the midgut of *G. m. centralis*, was not related to the presence of rickettsia in the midgut cells of the fly. These observations were later

confirmed by Shaw and Moloo (1991) who also found no positive correlation between *T. congolense* or *T. brucei* infections and the presence of RLOs in tsetse. These authors proposed that it was a combination of inter-related factors of both tsetse and parasite origin that determine the level of midgut infections of tsetse flies with *T. congolense* and *T. b. brucei*. Beard *et al.* (1993) using molecular techniques showed that bacterial symbionts previously designated as RLOs, possess characteristics different from those of the Rickettsiae family. At present the mechanism(s) controlling establishment and maturation of trypanosomes in the tsetse vector are not known.

2.4 Isolation of trypanosomes

Effective trypanosomiasis control requires an adequate understanding of trypanosome transmission cycles between *Glossina* species and animal hosts. However, this has been hampered by lack of reliable techniques for isolating trypanosomes from tsetse flies (Mihok *et al.*, 1994a) coupled with the difficulty of growing sufficient amounts of trypanosomes such as *T. b. gambiense* (Dukes *et al.*, 1989). This difficulty is due to low virulence for laboratory animals of *T. b. gambiense* (Gibson, 1986). Similarly, trypanosome species of the sub-genus *Duttonella*, such as *T. vivax* do not normally infect laboratory rodents (Dillman and Townsend, 1979) but require the expensive use of large animal hosts such as cattle or goats. Also, for many years, it has been observed that a large number of *T. congolense* field isolates do not infect laboratory rodents (Dukes *et al.*, 1991). *In vitro* techniques offer a useful alternative for maintaining and growing large amounts of trypanosomes under controlled conditions, in various media. Considerable progress has been made with the *in vitro* culture of trypanosomes. For example, *T. congolense* can now be cultured as animal-infective forms (Hirumi and Hirumi, 1984). It has also been demonstrated that *in vitro*-grown bloodstream forms and procyclic culture forms of *T. brucei* can complete their developmental cycle in *G. m. morsitans* (Schoni *et*

al., 1982). Cultured trypanosomes may retain their infectivity to tsetse flies and laboratory rodents, even after long-maintenance in cultures (Nyindo and Wellde, 1985). *In vitro* culturing of trypanosomes can be used for production of large numbers of various developmental stages of trypanosomes that can in turn be used for various purposes (ILRAD, 1990). These include; (1) *in vitro* screening of compounds for new therapeutic drugs or testing for drug resistance without interference of the host (Brun and Jenni, 1985; ILRAD, 1990),. (2) production of antigens for sero-diagnosis (ILRAD, 1990) and (3) biochemical studies such as antigenic variation for better understanding of the mode of action of various drugs (Vickerman, 1978; Fairlamb, 1982). However, *in-vitro* culturing of trypanosomes has some limitations. These include a limited number of developed systems that are simple and inexpensive (Brun and Jenni, 1985), and the inability of the technique to fully retain the parasite's natural characteristics. Long-term maintenance of trypanosomes in culture (Brun and Jenni, 1985) or in animal hosts (Dukes *et al.*, 1991) may lead to selection of trypanosome populations which are different from the strains found in natural hosts. Due to parasite adaptation to the new environment (cultures), 'altered' trypanosomes whose metabolic forms are very different from those of parasites growing in their natural hosts may be cultivated (Brun and Jenni, 1985).

Isolation of trypanosomes from tsetse flies may also be achieved by feeding gut-form (procyclics) into recipient susceptible flies, in which the trypanosomes are allowed to undergo cyclical development into mature forms. This technique, also known as the 'procyclic expansion', avoids natural selection of parasites due to long-term sub-passage in laboratory animals or culture as already discussed. Minter-Goedbloed *et al.* (1983) used laboratory-reared tsetse to isolate a reptile trypanosome stock designated 'F4' from naturally infected tsetse flies in Kenya. This was the first isolation of a non-salivarian trypanosome from the hindgut of the *morsitans* group. The procyclic expansion technique also enabled McNamara and Snow (1991) to characterise a new trypanosome belonging to the subgenus *Nannomonas* (*T. godfreyi*). The technique facilitated isolation of a variety of

Nannomonas trypanosomes from *Glossina* species in Kenya (Mihok *et al.*, 1994a). However, improvements are needed to make the procyclic expansion technique more applicable, especially under field conditions.

2.5 Characterisation of trypanosomes

Until recently, identification of *T. congolense* and other trypanosome species relied on the location of trypanosomes in the infected tsetse fly or on the morphology of the parasites in the infected animal host (Hoare, 1972; Dipeolu and Adam, 1974; Jordan, 1974; Otieno, 1983). The methods are labour-intensive, slow and have inherent limitations in sensitivity, specificity and morphological information. For example, *T. congolense* and *T. simiae*, the two species within the *Nannomonas* subgenus are morphologically indistinguishable and share the same developmental life cycle in the proboscis and the midgut (Hoare, 1970;1972). Moreover, these methods of identifying trypanosomes by morphology and site of development (Lloyd and Johnson, 1924) fail to distinguish mixed infections occurring in the same host or tsetse vector (Moloo *et al.*, 1982; Tarimo *et al.*, 1987). Furthermore, the gut-form trypanosomes belonging to different sub-genera are morphologically similar and therefore not distinguishable by morphological criteria or by site of development. These limitations led to the development of biochemical methods for detecting and identifying trypanosomes. In a brief investigation, Kilgour and Godfrey (1973) studied isoenzymes of bloodstream form trypanosomes using thin layer starch gel electrophoresis and demonstrated differences in enzyme mobilities between two stocks of *T. congolense*, thereby suggesting a potential value of using this technique in the characterisation of trypanosomes.

Young and Godfrey (1983) used isoenzyme electrophoretic analysis to reveal genetic polymorphism within *T. congolense* stocks obtained from different animal and tsetse fly hosts of Africa. The enzyme bands of *T. congolense* originating from humid

coastal areas of west Africa formed one group and *Trypanosoma congolense* from drier zones of Africa formed another group. The authors suggested that these major groupings could be a reflection of the adaptation of *T. congolense* parasites to different tsetse fly vector species occupying different ecological zones. Gashumba (1986), using electrophoretic analysis, demonstrated that *T. congolense* isolated from livestock at Kilifi cattle ranch on the Kenyan coast, comprised, a genetically distinct population, with different enzyme mobilities from other stocks isolated from West and East Africa. A similar finding was obtained by Majiwa *et al.* (1985). They showed that the repetitive DNA of *T. congolense* isolated from cattle exposed to natural infection at Kilifi was different from that of *T. congolense* isolated from other parts of East Africa. Knowles *et al.* (1988) confirmed these results through the combined use of enzyme electrophoresis and DNA hybridization technique. The significance of these genetic variations within *T. congolense* organisms in relation to the epidemiology of African trypanosomiasis remains unclear (Gashumba, 1986; 1990). Some biological characteristics such as response to trypanocides, infectivity to livestock, wildlife and *Glossina* species, for example, are unknown. Massamba and Williams (1984) developed a highly sensitive and specific (to the level of 10^4 organisms) procedure for identifying different *T. b. brucei* stocks and distinguishing *T. b. brucei* from *T. congolense* or *T. vivax*. The procedure could be performed on tsetse saliva or infected blood. Kukla *et al.* (1987) refined these DNA techniques to identify all trypanosome stages of *T. congolense, T. brucei* and *T. vivax* in various tsetse fly organs including the proboscides and midguts. A DNA probe is a pure, single stranded DNA fragment, between 15 and 1000 nucleotides long, used to detect the occurrence of its complementary DNA sequence in a mixture of nucleic acids obtained from cells (ILRAD Report, 1991). Pure single strand DNA is obtained by cloning from organisms of interest or synthesized by chemical means. As probes for identifying trypanosomes, DNA repeat units were used as these gave the DNA a high signal in identification (Gibson *et al.*, 1988). A range of these DNA probes became

available for use in identification of *Nannomonas* organisms (Gibson *et al.*, 1988). McNamara *et al.* (1989) in the Gambia used DNA hybridization technique to distinguish gut-form (procyclics) of *T. congolense* from *T. simiae* but the results were unsatisfactory because the majority of the DNA test samples made from field-captured infected tsetse flies could not be identified, inspite of having heavy *Nannomonas*-like trypanosomes.

Although detection by dot blot hybridization using species-specific DNA probes offered both sensitivity and specificity, its lower limit of detection was 100 trypanosomes or more. The technique was particularly useful for identification of trypanosomes in the fly midgut where they are present in large numbers and not in the proboscis where they are rarely heavy enough for dot blot hybridization (Masiga *et al.*, 1992). A more sensitive and specific technique based on amplification of repetitive DNA segments has now been developed which permits identification of a single trypanosome unequivocally (Masiga *et al.*, 1992). The use of DNA probes technique for trypanosome detection and characterisation has several advantages over other methods such as those which rely solely on microscopy. The technique is sensitive and specific (Massamba and Williams, 1984; Kukla *et al.*, 1987; Masiga *et al.*, 1992) and sample collection is also not tedious (Gibson *et al.*, 1988). Recently, it has been adapted for use under field conditions, as specimens can be stored dry under room temperature on nylon filters (Nyeko *et al.*, 1990). Use of DNA hybridization techniques has led to identification of new *Nannomonas* organisms such as the recently reported *Trypanosoma (Nannomonas) godfreyi* (McNamara *et al.*, 1994) and has also yielded valuable information on the distribution of *T. congolense* genotypes (Woolhouse *et al.*, 1993). To be of epidemiological value, the results from parasite characterisation using DNA analysis must be correlated with biological characteristics of the trypanosomes. The present investigation was not designed to explore the use of DNA hybridization technology in identifying trypanosome, rather, the technique only facilitated the study by providing accurately identified *T. congolense* genetic types.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Blood collection.

A. Jugular vein samples.

Blood samples from large animals (goats, cattle, pig, rhino, zebra, Thomson's gazelle and eland) were collected by jugular venipuncture. This was done by introducing an 18 G (goats, eland, Thomson's gazelle and zebra) or 14 G (cattle) needle into the vein and allowing the blood to flow into an evacuated tube containing heparin anticoagulant. Pig blood was collected from an aseptically severed neck. After collection, the blood was mixed thoroughly with the anticoagulant by gently shaking the tube.

Other samples of blood were defibrinated instead of mixing with an anticoagulant. This was accomplished by shaking freshly collected blood in a bottle containing glass beads. It was then kept at 4°C for 1-2 hours for the clotting materials to settle. The sample was then decanted into clean tubes. Defibrinated blood was preferred in investigations where the use of anticoagulants such as heparin could modify results.

B. Cardiac samples.

Cardiac blood was mainly collected from small laboratory animals such as mice, using a technique similar to that described by Lumsden and Evans (1979). Trypanosomes were harvested from an infected mouse at a rising parasitaemia of 10^6 to 10^7 trypanosomes per ml. To collect the blood, the mouse was anaesthetised inside a large glass desiccator containing cotton wool soaked in chloroform. This was covered with a second layer of dry cotton on top to prevent the animal from experiencing unnecessary suffering eye burns from liquid chloroform during anaesthesia. Once respiration was about

to cease, the mouse was pinned on a sterile 'dissection board', ventral side up and the entire abdominal surface sterilised by swabbing it with 70% alcohol. The diaphragm and the rib cage were cut to expose the heart without cutting the main blood vessels and avoiding piercing the wall of the heart. A 25 G needle fitted to a 4 ml syringe, whose inner sides were pre-wetted with heparin, was introduced into the heart and blood allowed to flow into the syringe with gentle suction.

3.2 Experimental procedures with mice.

A. Marking and maintenance of mice.

The experimental mice were individually marked with picric acid solution for identification (Lumsden and Evans, 1979). They were maintained on commercially prepared rodent pellets containing added vitamins and mineral salts (obtained from Unga Ltd, Nairobi). They were kept in groups of varying numbers (depending on the experiment), in plastic mice cages (35 cm x 20 cm x 20 cm). The cages were kept in a well ventilated, clean room.

B. Immunosuppression of mice.

Many strains of *T. congolense*-like organisms tend to produce low levels of parasitaemia in laboratory animals (Dukes *et al.*, 1991). Parasitaemia is usually increased by suppressing the capacity of the animal to make an effective immune response. In these studies immunosuppression was done one day before infection, by injecting freshly-prepared cyclophosphamide solution into their peritoneal cavity at a dose of 200 mg per kg live body weight.

3.3 Experimental procedures with tsetse.

A. Infection and dissection.

Tsetse in groups of 50 per Polyvinyl Cage (PVC) (PLATE 1) were infected with trypanosomes through a silicone membrane (Mews *et al.*, 1977). Infected flies were maintained for 10 days for midgut infections and maintained for 28-30 days for hypopharynx (mature) infections. The tsetse flies were dissected between days 3 and 21 after infection for midgut infections and on days 28-30 for mature infections, depending on the experiment.

The flies were briefly immobilised at 4°C in the fridge and their wings and legs were removed to facilitate dissection. Dissections of proboscides and midguts were made in phosphate-saline-glucose (PSG), pH 7.0 as described by Murray *et al.* (1983). The dissected parts were examined at 400x magnification in 20 μl PSG for presence of trypanosomes.

PLATE 1. Polyvinyl cage (PVC) for holding tsetse.

B. Rearing and maintenance

Glossina m. centralis were reared in the International Centre of Insect Physiology and Ecology (ICIPE) insectary, Nairobi. The flies were maintained at a temperature of 25 $\pm$ 1°C, a relative humidity of 70-80% and 12 h light:12 h dark photoperiod. *Glossina m. centralis* stock established at ICIPE for this study was obtained from the ILRAD colony in 1990; the ILRAD stock originated from Shinyanga, Tanzania.

3.4 Origin of *Trypanosoma congolense* isolates.

Trypanosoma congolense stocks were isolated from Tsavo National Park, Kilifi cattle Ranch on the Kenyan coast, the Lambwe Valley and Rusinga Island in Nyanza, western Kenya. In Kenya, *G. pallidipes* is the predominant *Glossina* species as shown by various researchers in Tsavo (Mihok *et al.*, 1992a), in the Lambwe Valley (Allsopp and Baldry, 1972; Turner, 1987) and on the Kilifi cattle Ranch (Paling *et al.*, 1987). These authors described the areas in detail and provided substantial information on wildlife species in each of them.

3.5 Data analysis

The computer programme Lotus (1-2-3) was used for recording data, whereas Slide Write was used for graphics. The Statistical Analysis System (SAS) was used for Analyses of Variance (ANOVA) using the Student-Newman-Keuls (SNK) multiple range test to determine differences among means. Percentages were transformed by the arcsin transformation to equalise variances before applying ANOVA (Sokal and Rolf, 1981). The Chi-square and Fisher's Exact Test were used to determine differences between proportions.

CHAPTER 4

ISOLATION, CLONING AND IDENTIFICATION OF *Trypanosoma congolense.*

4.1 INTRODUCTION

Trypanosoma congolense types can be divided into four genomic subgroups based on differences in repetitive DNA sequences (Young and Godfrey, 1983; Majiwa *et al.*, 1985; Garside *et al.*, 1994). These groups are: Savannah, West African Riverine/Forest, Tsavo and Kilifi. Recently, a further variant from the Gambia has been characterised; biochemical and host range data have suggested it is a new species of *Nannomonas,* and hence, it has been named *T.* (*Nannomonas*) *godfreyi* (McNamara and Snow, 1991 and McNamara *et al.*, 1994).

At present the distribution of the *T. congolense* types in Africa is unclear (Gashumba, 1990). Similarly, characteristics such as responses to trypanocides and infectivity to livestock, wildlife or tsetse flies have not been studied (Gashumba, 1990). Therefore, field isolates of a variety of *T. congolense* genotypes from tsetse and hosts are needed to facilitate studies. The standard method for isolating trypanosomes involves inoculating them into a susceptible host where they multiply and are harvested. Unfortunately, many field *Nannomonas* trypanosomes are 'lost' through their failure to infect laboratory animals (Dukes *et al.*, 1991). These 'lost' trypanosomes may represent a 'pool' of epidemiologically useful information (Mihok *et al.*, 1993) as indicated by the recent discoveries of new *Nannomonas* trypanosomes (McNamara *et al.*, 1994).

New methods for field isolation of trypanosomes are now available (Dukes *et al.*, 1989; McNamara and Snow, 1991), with the choice of a particular method being dictated by factors such as adaptability to field use and costs. Recently, Mihok *et al.* (1994a) showed that gut-form trypanosomes can be passaged from one tsetse species to another with a high level of success. In this study, gut-form transfer (procyclic expansion) and

mouse inoculation were used for field isolation of *T. congolense*. These isolates were further cloned in mice. Cloning is essential because trypanosome isolates even from single sources may contain representatives of several different populations. For interpretation of trypanosome behaviour, it is essential to work with pure, homogeneous material, preferably, of known genetic composition.

4.2 MATERIALS AND METHODS

A. Isolation from cattle and tsetse

Jugular blood (see Chapter 3) was examined using the buffy coat concentration technique (Murray, 1977). Trypanosomes were identified by their characteristic behaviour of adhering to one another (autoagglutination) in wet blood smear (Shar-Fischer and Say, 1989) and also by their morphological features in thin blood smears stained with 10% Giemsa solution (Hoare, 1970; 1972). An antigen detection latex test (obtained from Brentec Diagnostics, Nairobi, Kenya) was used to detect infected cattle as follows: 50 µl of test plasma was mixed with 50 µl of latex reagent on a glass slide and the mixture mechanically stirred for 5 minutes. Agglutination of the sample indicated previous exposure of the animal to trypanosome infections. The results were scored by visual inspection as: (i) strong positive (ii) medium and, (iii) weak positive. The presence of trypanosomes in blood samples was confirmed through microscopic examination of the buffy coat at 400x magnification. Blood samples showing low parasitaemias of *T. congolense*-like trypanosomes as indicated by microscopy were inoculated into mice to enable them to multiply, those that grew in mice were cryopreserved in 10% glycerol for subsequent cloning and biochemical identification with PCR.

Fly isolates were obtained from *G. pallidipes* Austen trapped in Ruma National Park in the Lambwe Valley, Kenya, using NG2G and Biconical (Laveissiere) traps described by Brightwell *et al.* (1991) and FAO (1992). Chemical baits comprising of acetone and cow urine obtained from local zebu cattle and stored three weeks, were also used for attracting and catching the insects as described by Dransfield *et al.* (1986). Traps were set 200 metres apart and left in the field for the whole sampling period. Trap inspection and emptying was done daily at 09.00 hours.

The mouthparts and guts of tsetse were examined at 400x magnification for the presence of trypanosomes as described earlier. Initial identification was based on the

location of infection in the fly (Lloyd and Johnson, 1924) as follows: proboscis only (*Duttonella* subgenus, *T. vivax*), proboscis and gut (*Nannomonas* subgenus, *T. congolense* or *T. simiae*), proboscis, gut and salivary glands (*Trypanozoon* subgenus, *T. brucei*, *Pycnomonas* subgenus, *T. suis*), gut only (immature *Nannomonas*, *Trypanozoon* or *T. grayi*). 'Gut only' parasites were cryopreserved in goat blood and cow serum in 10% glycerol for procyclic expansion experiments. An aliquot of gut-forms was loaded onto a Hybond-N$^+$ membrane for identification. Proboscids showing *Nannomonas* infections were macerated individually in 200 μl phosphate-saline-glucose and inoculated into Balb/c mice that had been immunosuppressed with cyclophosphamide solution. The mice were examined for trypanosome infection three times a week, for a month, starting three days after infection. Cardiac blood was obtained from infected mice as described in Chapter 3 at rising parasitaemia of over 10^5 trypanosomes per ml (Herbert and Lumsden, 1976) and isolates were cryopreserved in 10% (v/v) glycerol for subsequent cloning.

Trypanosomes were transferred into *G. m. centralis* to isolate trypanosomes according to the method of Mihok *et al*. (1994a). Briefly, 15 to 20 infected guts of flies were pooled and macerated in 200 μl phosphate-saline-glucose, pH 7.0 and mixed with 5 ml freshly defibrinated goat blood, using a vortex mixer. Cholesterol or D(+)-glucosamine was added to the infective feed (final concentration in blood of 0.01 M) in order to increase infection rates. Experimental flies were then promptly fed on the blood mixture. In double cholesterol feeds, flies were fed on additives during the first two bloodmeals and then maintained on a rabbit until dissected for examination of trypanosomes, 21 days later.

B. Viability of procyclics.

Gut-forms were obtained by macerating 17 infected guts of *G. m. centralis* (10 days post-infection) in PSG (100 μl), pH 7.0, in a vortex machine. Aliquots of the trypanosome suspension were each mixed with the following to a final volume of 500 μl: goat blood, goat serum, foetal calf serum and goat blood with a 75% Packed Cell Volume

including cholesterol at final concentration of 0.01 M. Each sample was divided into two equal parts. One part was heated for 45 minutes at 56°C in a water bath to inactivate complement, whereas the other was left unheated. The procyclic mixtures were left at room temperature (25 ± 3 °C) and examined after various time intervals for 24 hours at 400x magnification. Numbers of surviving trypanosomes were recorded accordingly. The motility was also assessed at 24 hours to check for the presence of sluggish trypanosomes.

C. Cloning

Bloodstream form trypanosomes were cloned using the hanging-drop method (Murray *et al.*, 1983) in cyclophosphamide-immunosuppressed Balb/c and Swiss mice.

D. Characterisation

Dot-blots of gut-infections were made on Hybond-N$^+$ nylon membranes (Amersham). These were air-dried, denatured (Nyeko *et al.*, 1990) and stored between filter papers in polyethylene bags at -20°C in a deep freezer. For each sample of gut-form trypanosomes, an aliquot was also cryopreserved in 10% glycerol.

Trypanosoma congolense La1, La2 and La3 were isolated from zebu cows at Gwassi, Lambwe Valley, Kenya. Each isolate was passaged once in mice before cloning. Ng5 was cloned from an isolate at Tsavo National Park from *G. pallidipes* and is of the Savannah DNA type (Mihok *et al.*, 1992a). Uku stocks were isolated from Ukunda at the Kenyan coast from cattle. All the stocks used in this study were characterised by Polymerase Chain Reaction to confirm DNA types. IL 3900 is a *T. congolense* West African Riverine/Forest clone of a stock isolated from a dog in Burkina Faso and maintained at the International Laboratory for Research on Animal Diseases.

4.3 RESULTS

A. Isolation from cattle and tsetse

Gut-forms of Ng5 passaged into *G. m. centralis* produced midgut infections in four out of five attempts. Infection rates were generally increased through the use of additives. In particular, cholesterol at a concentration of 0.01 M in blood, fed to flies in the first two bloodmeals increased midgut infection rates significantly compared to controls (Table 1 and Fig. 2). The increase was true for both sexes, but more dramatic in males (N = 98, X^2 = 43.34, P = 0.000) than in females (N = 99, X^2 = 8.45, P = 0.004 and Fig. 2). D(+)-glucosamine at a concentration of 0.01 M in the infective blood, fed to flies once, also increased midgut infection rates of Ng5 (Table 1).

The overall prevalence of *T. congolense*-like infection determined by microscopy was 1.3% in *G. pallidipes* and 4.2% in cattle (Table 2; Fig. 3). However, an antigen test showed that 40% of cattle examined at Lambwe Valley and 53% examined at Rusinga Island were exposed to *T. congolense* infections (Figs. 4 and 5; Appendices 1 and 2).

Some tests of Cholesterol and D(+)-glucosamine additives with gut-forms of field isolates were mostly unsuccessful (Table 3) despite good viability following cryopreservation. Neither *G. m. morsitans* nor *G. pallidipes* supported trypanosome infection when passaged with cryopreserved gut-forms from the field (Table 4). Twenty percent of tsetse isolates grew in immunosuppressed mice whereas 60% of cattle isolates produced a detectable parasitaemia in the mice (Table 5).

B. Viability of procyclics.

The Ng5 stock of *T. congolense* survived for the whole observation period of 24 hours at room temperature, maintaining motility and numbers of between 10^3 to 10^4 trypanosomes per ml in the various blood mixtures (Table 6).

C. Cloning

Cloning was successful in all four trials. Balb/c mice were significantly more susceptible to field isolates than Swiss mice (Table 7).

D. Characterisation

PCR tests indicated that all the four trypanosome isolates from the Lambwe Valley and from the Kenyan Coast were Savannah *T. congolense* types (Table 8). One of the three isolates from Lambwe (La2) and the isolate from the Kenyan coast (Uku1) may have also contained *T. brucei*, according to PCR. However, *T. brucei* could not be identified in these stocks or their clones through microscopic examination.

Table 1

Attempts to passage 12-day- and 22-day-old T. congolense *Ng5 procyclics into*
G. m. centralis **in Chol 0.01 M   and   GlcN 0.01 M.**

Blood	Sex	Fed	Diss.	% Inf	P	% Surv.
22-day procyclic						
Goat	M	125	119	4		95
Goat + Chol	M	200	159	46	0.000	80
Goat	F	125	82	13		66
Goat + Chol	F	125	91	18	0.051	73
Goat	F	168	114	17		68
Goat + GlcN	F	120	92	44	0.000	77
12-day procyclics:						
Goat	M	200	120	0		60
Goat + Chol	M	200	127	2	0.167	63
Goat	F	100	58	0		58
Goat + Chol	F	100	60	0		37

Goat: Defibrinated goat blood; **GlcN**: D(+)-glucosamine; **Chol**: cholesterol; **M**: Male;
F: Female; **% Inf.**: Percentage of tsetse infected on day 10; **% Surv.**: Percentage of tsetse
surviving to day 10; **Diss**: dissected; *P*: probability.
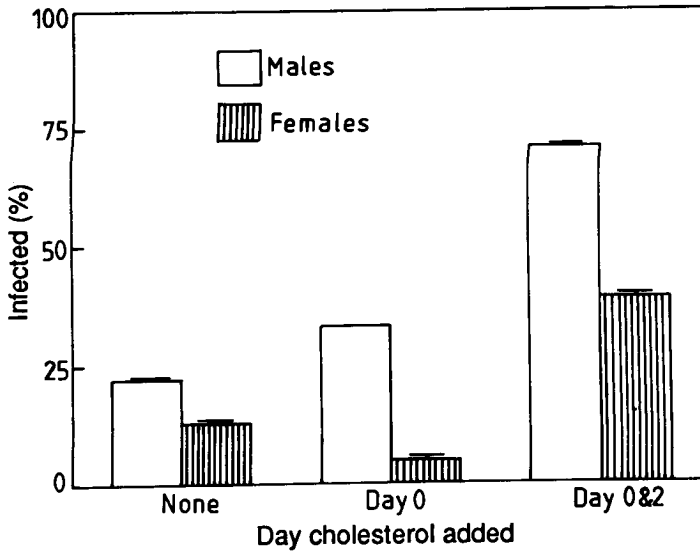
Figure 2 Effect of single and double cholesterol feeds on infection rates in

G. m. centralis **passaged with** *T. congolense* **Ng5 gut-forms.**

(Tsetse were fed on goat blood mixed with cholesterol either on day 0 (single feed) or on days 0 and 2 after infection (double feed). Control flies received blood mixed with trypanosomes without additives between day 0 and 2. Tsetse were dissected 10 days after infection).

Table 2

Trypanosome infection rates(%) in *G. pallidipes* **and in cattle determined during field trial 1 (May, 1992) and trial 2 (June, 1992) in the Lambwe Valley, Kenya.**

	Number of tsetse infected			
Type of infection	M	F	M + F	Number of cattle infected
Nannomonas	5	7	12	1 5
Duttonella	26	41	67	5
T. brucei	0	0	0	1
Immature	4	2	6	-

G. pallidipes: **N** = 891 ;   Cattle: **N** = 357;   **F**: Female;   **M**: Male.
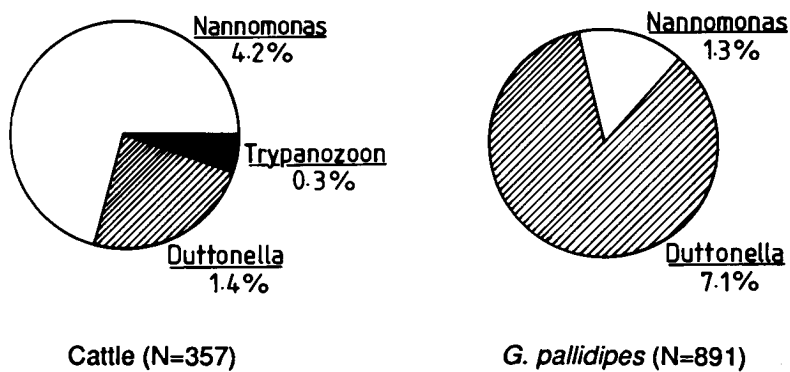
Figure 3   Trypanosome infection rates(%) in *G. pallidipes* and cattle during field trial 1 (May, 1992) and field trial 2 (June, 1992) in the Ruma National Park, Lambwe Valley, Kenya.
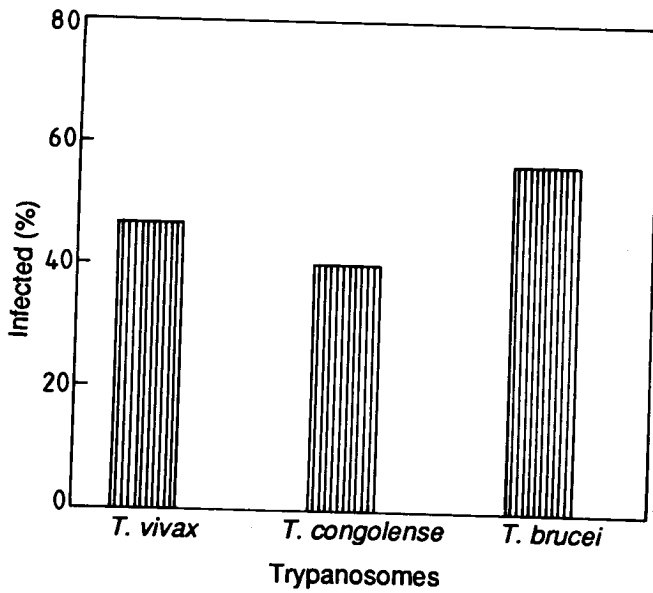
Figure 4 Trypanosome infections in cattle determined by microscopy of buffy coat and antigen detection (latex agglutination) test (shaded bars) of plasma during trial 3 at the Lambwe Valley (March, 1994).
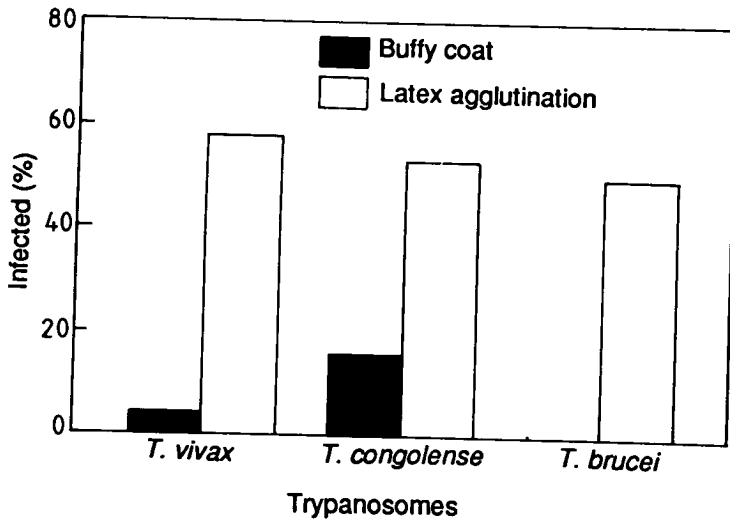
Figure 5 **Trypanosome infections in cattle determined by microscopy of buffy coat and antigen detection (latex agglutination) test of plasma during trial 3 at Rusinga Island (March, 1994).**

Table 3

Isolation of immature trypanosomes from wild *G. pallidipes* **through passage of cow-cryopreserved (cryoC) and goat-cryopreserved (cryoG) procyclics into** *G. m. centralis* **fed on either Chol 0.01 M or GlcN 0.01 M.**

Treatment	Sex	Fed	Dissected	% Infected	% Surv.
<u>Stock1</u>					
CryoG, Goat	M	50	30	24	60
CryoG, Goat + GlcN	M	50	16	12	32
CryoG, Goat + GlcN	M	50	4	0	8
<u>Stock 2</u>					
CryoC, Goat	M	100	70	0	70
CryoC, Goat + Chol	M	100	46	0	46
CryoC, Goat + Chol	F	100	59	0	59
<u>Stock3</u>					
CryoC, Goat	M	50	27	0	54
CryoC, Goat + Chol	M	100	61	0	61
CryoC, Goat + Chol	F	100	58	0	57

Goat: Defibrinated goat blood; **GlcN:** D(+)-glucosamine; **Chol:** cholesterol; **M:** Male; **F: Female**; **% Inf.:** Percentage of tsetse infected on day 10; **% Surv.:** Percentage of tsetse surviving to day 10; **Diss.:** dissected.

Table 4

Isolation of immature trypanosomes from wild *G. pallidipes* **through passage of cryopreserved goat-(cryoG) and foetal calf serum-procyclics (cryoS) into** *G. m. morsitans* **and** *G. pallidipes* **fed on either Chol 0.01 M or GlcN 0.01 M.**

Treatment	Sex	Tsetse Fed	Tsetse Dissected	Infected	% Surv.
G. m. morsitans					
CryoG, Goat	M	50	40	0	80
CryoG, Goat	F	50	34	0	68
CryoG, Goat + GlcN	M	50	40	0	80
CryoG, Goat + GlcN	F	50	33	0	74
CryoG, Goat + Chol	M	50	38	0	88
CryoG, Goat + Chol	F	50	31	0	62
G. pallidipes					
CryoG, Goat	M	50	18	0	51
CryoG, Goat + Chol	F	50	18	0	37
CryoS, Goat + Chol	F	50	0	0	0

Goat: Defibrinated goat blood; **GlcN:** D(+)-glucosamine; **Chol.:** cholesterol; **M:** Male;

F: Female; **% Surv.:** Percentage of tsetse surviving to day 10.

Table 5

Isolation of *T. congolense*-**like parasites from wild** *G. pallidipes* **and cattle through mouse inoculation at the Lambwe Valley and Mbita Point, South Nyanza (May-June, 1992 and March, 1994).**

Source	N	Place*	Stocks	Number of isolates	(%)
G. pallidipes	1368	Lambwe	24	5	(20.8)
G. f. fuscipes	516	Mbita	0	0	(0.0)
Cattle	392	Lambwe	10	6	(60.0)
Cattle	26	Rusinga	4	0	(0.0)

Mbita: Mbita point;   **Place*:** Place of isolation;   **N**: Number of cattle or tsetse examined.

Table 6

Survival of *T. congolense* **Ng5 gut-forms incubated in different blood mixtures at 25 ± 3°C.**

	Number of trypanosomes / Number of fields searched				
Treatment	**Start**	**2 h**	**4 h**	**19 h**	**24 h**
Gb, Unh	1/28	1/10	1/20	1/14	1/41
Gb, Ht	1/10	1/10	1/25	1/5	1/5
GS, Unh	3/10	1/15	1/5	1/4	1/22
GS, Ht	2/10	1/10	1/9	1/8	1/9
FCS, Unh	1/10	1/10	1/10	1/5	1/16
FCS, Ht	1/10	1/10	1/10	1/12	1/14
75% PCV, Unh	1/11	1/10	1/12	1/11	1/15
75% PCV, Ht	1/10	1/13	1/10	1/18	1/10
Gb + Chol, Unh	1/24	0/30	1/9	1/26	1/30
Gb + Chol, Ht	1/10	1/13	1/10	1/18	1/17

Gb: Goat;   **GS:** Goat serum;   **PCV:** Packed Cell Volume;   **Ht**: Heated at 56°C for 30 minutes;   **Unh:** Unheated;   **h**: hour;   **Chol:** cholesterol; **FCS:** Foetal Calf Serum.

Table 7

Infection rates of *T. congolense* clones (La1, La2, La3 and Uku1) and prepatent periods between inoculation and detection of parasitaemia in Balb/c and Swiss mice.

Clone	Mice	Inoc.	Inf.	%	X^2	*P*	PPP Mean ± SE
Trial 1							
La1	Swiss	15	0	0.0			
La1	Balb/c	15	9	60.0	12.86	0.000	12 ± 0.14
Trial 2							
La2	Swiss	15	0				
La2	Balb/c	15	1	6.7	1.03	0.030	11
Trial 3							
La3	Swiss	15	0	0.0			
La3	Balb/c	15	4	26.7	4.62	0.030	18 ± 3.00
Trial 4							
Uku 1	Swiss	15	1	6.7			
Uku 1	Balb/c	15	9	60.0	9.60	0.000	13 ± 2.20

Inoc[*]: Syringe inoculation of mice with trypanosomes; **Inf.:** infected with trypanosomes; **PPP**: Prepatent period; X^2: Chi-square Test; **SE**: Standard error; *P*: Probability.

Table 8

Molecular characterisation of *Trypanosoma congolense.*

			Identity by PCR			
Stock	**Origin**	**Host**	**Tc. Savannah**	**Tc. Kilifi**	**Tc. Forest**	*Trypanozoon*
La1	Lambwe	Cow	+	-	-	-
La2	Lambwe	Cow	+	-	-	+
La3	Lambwe	Cow	+	-	-	-
Uku1	Coast	Cow	+	-	-	+
Ng5	Tsavo	Gp	+	-	-	-

La: Lambwe *Trypanosoma congolense*; **Ng5**: Ngulia *T. congolense*; **Uku**: Ukunda *T. congolense*; **Tc. Sav:** Savannah *T. congolense*; **Tc. Forest**: Forest/Riverine *T. congolense*; **Tc Kilifi**: Kilifi *T. congolense*; **Gp**: *G. pallidipes*; **PCR**: Polymerase Chain Reaction.

4.4 DISCUSSION

Isolation of *T. congolense* through procyclic expansion into tsetse was achieved only in *G. m. centralis*, not in *G. m. morsitans* and *G. pallidipes*. Differences in overall susceptibility of flies to *T. congolense* infections probably accounted for this failure since the cryopreserved trypanosomes used for procyclic passage showed excellent viability. Moloo *et al.* (1992a) compared laboratory *G. m. centralis* and *G. pallidipes* as vectors of *T. congolense*, *T. brucei* and *T. vivax*. They found that *G. m. centralis* with an infection rate of 22.5% was a better vector of *T. congolense* than *G. pallidipes* with an infection rate of 8.5% for a stock from a lion in Tanzania. Also, *G. m. centralis* (18.4%) showed a higher susceptibility than *G. pallidipes* (2.7%) for a stock of *T. brucei* from Tanzania. However, *T. vivax* infections in *G. m. centralis* and *G. pallidipes* were similar. More recently, Moloo (1993b) has compared the susceptibility of laboratory *G. pallidipes* populations originating from different geographical areas of Kenya to *T. congolense*. He found that *G. pallidipes* from Nguruman, Rift Valley Province was more susceptible to *T. congolense* than *G. pallidipes* from Shimba Hills, Coast Province. Numerous studies have shown that *G. m. morsitans* is relatively resistant to infection (reviewed by Maudlin, 1991).

Maudlin and Ellis (1985) reported an association between bacterial endosymbionts and susceptibility to *T. congolense* infections in teneral flies. The mechanism proposed involves the production by endosymbionts of chitinase (Maudlin, 1991) which degrades chitin of the peritrophic membrane in the larval-pupal stage to N-acetyl-glucosamine and small amounts of glucosamine. These amino sugar residues then bind lectins that would otherwise agglutinate and kill trypanosomes present in the first bloodmeal. D(+)-glucosamine fed to tsetse infected with *T. congolense* and *T. b. brucei* can significantly increase gut infection rates compared to flies fed on blood without the amino sugar (Maudlin and Welburn, 1987). It was also shown that the secretion of lectins in tsetse guts

was stimulated by a bloodmeal, since newly-emerged flies possessed lower lectin levels than blood-fed flies. The higher susceptibility to trypanosome infections reported in newly-emerged and starved tsetse was attributed to low lectin levels in these flies.

The peritrophic membrane (Lehane and Msangi, 1991), trypano-agglutinins and trypanolysins (Stiles *et al.*, 1990), and tsetse digestive enzymes (Osir *et al.*, 1993) are also thought to be important in determining the establishment of trypanosomes in the gut. It is not known which of these many factors might have determined the successful establishment of gut-forms transferred into *G. m. centralis* in this study. However, results presented in this investigation indicate the potential use of *G. m. centralis* in isolating field *T. congolense* and therefore corroborate previous findings (Mihok *et al.*, 1994a) that gut-form trypanosomes can be 'transferred' from one tsetse species to another with reasonable success.

The procedure used in this study to expand field *T. congolense* by gut-form transfer was based on three phenomena known to increase trypanosome infection in tsetse flies. These are: use of goat blood for feeding (Mihok *et al.*, 1993); use of a susceptible vector such as *G. m. centralis* (Moloo *et al.*, 1992a); addition of a midgut lectin inhibitor, D(+)-glucosamine (Maudlin and Welburn, 1987; Maudlin, 1991; Welburn *et al.*, 1994; Mihok *et al.*, 1992b) or addition of cholesterol (Mihok *et al.*, 1994a). Cholesterol addition in the first two bloodmeals to *G. m. centralis* increased Ng5 *T. congolense* infections significantly compared either to a single cholesterol feed or goat blood alone. Tsetse survival in these double cholesterol feeds was generally good, ranging from about 60.0% to 80.0%. In four attempts with untyped *Nannomonas* stocks from Lambwe, cholesterol did not appear to facilitate isolation as no parasite grew.

Maudlin *et al.* (1984) showed that complete removal of serum from cow blood can result in over 90% gut infection of *T. congolense* in *G. m. morsitans* fed on procyclic culture forms. Serum-free horse blood similarly produced a significant increase in midgut

infections of *T. b. rhodesiense* in *G. m. morsitans* infected with bloodstream forms (Gingrich *et al.*, 1982). It appears from these investigations that the factor(s) responsible for preventing midgut infections from becoming established reside(s) mainly in host serum. However, recent data also indicate that red blood cells contain components which affect midgut establishment of *T. congolense* (Olubayo *et al.*, 1994).

The presence of cholesterol in the first two goat bloodmeals including the infective feed increased midgut infection rates in *G. m. centralis* fed on gut-forms for some but not all trypanosome stocks. Cholesterol is a major component of cell membranes (Johnson *et al.*, 1991). Venkatesan and Ormerod (1976) observed accumulation of cholesterol *in vivo* as pleomorphic *T. b. rhodesiense* transformed from slender to stumpy forms in the blood of animals. The stumpy forms are considered to be biochemically pre-adapted for survival in the tsetse gut (Vickerman, 1970). Cholesterol is poorly digested by non-infected tsetse flies, with over 90% excreted (Langley *et al.*, 1987). At present there is no clear understanding of how cholesterol facilitates survival of procyclic forms (Mihok *et al.*, 1994a).

D(+)-glucosamine increased infection rates of Ng5 but failed to do so for other Savannah type stocks from Lambwe. In previous studies glucosamine increased infection rates of three field isolates from another locality in Kenya (Mihok *et al.*, 1994a). There is little information on the use of glucosamine in gut-form transfer experiments for comparison with the present results. Most investigators (Maudlin and Welburn, 1987; Welburn *et al.*, 1994 and Mihok *et al.*, 1992b) have used glucosamine to increase infection rates with bloodstream forms and not gut-forms. D(+)-glucosamine increased trypanosome infection rates but also caused fly mortality. Tsetse mortality often occurred 2 to 5 days after infection. Mihok *et al.* (1994a) also reported fly mortality with glucosamine in *G. m. centralis* fed on gut-forms. On one occasion tsetse mortality was attributed to bacteria suspected to have originated from rabbits used for maintaining the

infected flies. Dukes *et al.* (1989) lost field stocks due to bacterial contamination during field isolation from *G. m. morsitans* by procyclic expansion in culture. Bacterial contamination seems to be an important factor that may limit the successful isolation of trypanosomes by the gut-form transfer procedure, particularly when glucosamine is used.

Various methods of trypanosome isolation from tsetse seem feasible. Minter-Goedblood *et al.* (1983) isolated a non-salivarian, reptilian trypanosome designated 'F4' from the hindgut of a naturally infected *G. pallidipes* by feeding gut-forms to *G. m. morsitans*. A synthetic blood diet provided the infective feed. Dukes *et al.* (1989) isolated 75% of *T. b. gambiense* from sleeping sickness patients in Cameroon by feeding cryopreserved isolates in horse blood to laboratory-reared *G. m. morsitans* followed by cultivation *in vitro* of the procyclic forms from the infected fly midguts. McNamara and Snow (1991) isolated 68% of field isolates through the combined use of short-term *in vitro* cultivation followed by procyclic expansion in a susceptible strain of *G. m. morsitans*. The procyclic expansion technique enabled them to characterise a new trypanosome species, *Trypanosoma (Nannomonas) godfreyi* (McNamara *et al.*, 1994). Recently, 70% of field *T. congolense* and *T. simiae* stocks were isolated using susceptible *G. m. centralis* fed on various blood products (Mihok *et al.*, 1994a).

The choice of blood media for cryopreserving *T. congolense* types does not seem to be a critical factor. Procyclic trypanosomes incubated in various blood mixtures survived and retained viability for more than a day at room temperature ($25 \pm 3°C$). However, Adams (1931) reported that fresh sera of a number of mammals, birds and reptiles was trypanocidal to gut-forms of *T. b. rhodesiense* and *T. b gambiense*. The trypanocidal activity in fresh serum was attributed to complement. It is possible that this complement-mediated factor in fresh sera, trypanocidal to *T. b. rhodesiense*, is species-specific.

The 20% success rate obtained with mouse inoculation of fly isolates in this study is similar to results from other field studies. For example, Mihok *et al.* (1992a) isolated

24% of the *Nannomonas* organisms found in *G. pallidipes* from Tsavo West National Park. Sixty percent of *T. congolense* organisms from cattle grew in mice. Dillman and Townsend (1979) managed to grow all the *Trypanozoon* stocks originating from various wildlife species in the Luangwa Valley of Zambia in mice. However, only 42% of *T. congolense*-like organisms from wildlife produced detectable parasitaemias. Failure to produce *Nannomonas* infections in mice was at first attributed to using too few mice per sample(3). However, for one lion blood sample, ten mice were used but no parasite was isolated. The inability of some *Nannomonas* organisms to develop in mice is an important limitation that has long been recognised (Dukes *et al.*, 1991).

The possible cryptic presence of mixed infections of *T. congolense* and a *Trypanozoon* in some clones is puzzling. Majiwa and Otieno (1990) reported a few mixed infections of *T. congolense* types and *T. b. brucei* infections in 74 gut infections from 1086 *G. pallidipes* dissected in the Ruma National Park. Also, mixed infections of Kilifi and Savannah type *T. congolense* were observed in some of the *G. pallidipes*. Their finding of the Kilifi type in the Lambwe Valley was a surprise since previous studies (Gashumba *et al.*, 1988) suggested that Kilifi parasites were confined to the coastal region of Kenya. The Kilifi *T. congolense* types were first isolated by ILRAD scientists from 'sentinel' cattle exposed to natural infection, on a ranch in Kilifi, at the Kenyan Coast, where *G. austeni* is the resident tsetse fly species. A new *T. congolense* type has been reported in a population of *G. pallidipes* captured in the Tsavo National Park (Majiwa *et al.*, 1993a). Another new *Nannomonas* trypanosome, *T. (N) godfreyi* has been isolated from *G. m. submorsitans* in the Gambia (McNamara and Snow, 1991; McNamara *et al*., 1994). The isolation of *T. congolense* genotypes and similar *Nannomonas* trypanosomes from different tsetse species i.e. *G. austeni, G. pallidipes* and *G. m. submorsitans,* suggests that the species of vector is not a barrier to transmission of these trypanosome genotypes to mammalian hosts. The studies of Majiwa and Otieno (1990) suggest a large diversity of *T. congolense* types in the Lambwe Valley. As various *T. congolense*

genotypes are isolated from the field and described accurately, a need arises to investigate their biological characteristics in various hosts. This aspect will be covered in Chapter 5.

CHAPTER 5

HOST BLOOD EFFECTS ON MIDGUT INFECTIONS OF
Trypanosoma congolense IN TSETSE

5.1 INTRODUCTION

The developmental cycle of *Trypanosoma congolense* involves a vertebrate and a tsetse fly host. The transition from the mammalian host to tsetse is critical for the trypanosome since it must adapt quickly to the new environment inside the tsetse gut for it to survive. Gut establishment involves a complex interaction between the fly, endosymbionts and the trypanosome itself (Maudlin and Ellis, 1985; Maudlin and Welburn, 1988). The process is mediated by several factors such as: (1) midgut lectins (Maudlin and Welburn, 1987; 1988; Welburn *et al.*, 1989) (2) trypanoagglutinins and trypanolysins (Ibrahim *et al.*, 1984; Ingram and Molyneux, 1991) and (3) digestive proteases (Stiles *et al.*, 1991; Imbuga *et al.*, 1992; Osir *et al.*, 1993). Some researchers have proposed that the peritrophic membrane might influence the development of midgut form trypanosomes (Willet, 1966; Okolo *et al.*, 1988; Lehane and Msangi, 1991). Similarly, in mosquitoes the peritrophic membrane serves as a limiting rather than an absolute barrier to development of malaria parasites (Ponnudurai *et al.*, 1988). The membrane possesses receptor sites that determine vector competence in tsetse and mosquitoes (Okolo *et al.*, 1988; Huber *et al.*, 1991).

The hosts on which tsetse feed greatly influence the type and rate of infection in flies. Thus, hosts are major determinants in the epidemiology of African trypanosomiasis. The relationship between the proportion of bloodmeals taken from bovids and a high infection rate with *T. vivax* is well established (Jordan, 1965; Snow *et al.*, 1988). However, there is little information on the influence of host blood on various *T. congolense* genotypes, although studies have been performed to determine the influence of

host blood on trypanosome establishment in tsetse (Moloo, 1981; Mihok *et al.*, 1991; Mihok *et al.*, 1993; Olubayo *et al.*, 1994). The studies reported in this chapter were conducted to investigate, among other things, the effect on establishment of molecular types of *Trypanosoma congolense* when mixed with blood of various animal species. Understanding host influence on trypanosome infections in the vector is essential in formulating effective disease control strategies.

5.2 MATERIALS AND METHODS

A. Host effects on midgut infections.

Glossina m. centralis were membrane-fed on fresh blood from a zebra, Thomson's gazelle, black rhino, eland, goat, Boran cow, pig and donkey mixed with *T. congolense* Ng5 and K60/1 stock. The flies were dissected on day 28 to check the presence of gut-form trypanosomes. In another trial, the influence of host blood on midgut *T. congolense* (K60/1) infection rates was studied in *G. m. centralis* using goat and eland blood by varying the sequence of blood feeding as: (1) Double goat; fed on infective goat blood during the first feed (day 0) and fed again on non-infected goat blood on day 3. (2) Double eland; fed on infective eland blood on day 0 and fed on non-infective eland blood on day 3. (3) Eland followed by goat and (4) Goat followed by eland.

B. Effect of GlcNAc and Chol on infections.

To compare the effect of chemical compounds on K60/1 and Ng5 *T. congolense* midgut infection rates, tsetse flies were fed during the first infective feed on fresh blood mixed with either cholesterol (Chol) alone or in combination with GlcNAc at a final concentration of 0.05 M and 0.01 M for GlcNAc and Chol respectively. Control flies were fed on blood mixed with trypanosomes without chemicals. Flies were maintained on a new rabbit changed weekly to avoid re-infections. Tsetse were dissected on Day 10 to detect presence of trypanosomes in the gut as described in Chapter 3.

C. Patterns of *Trypanosoma congolense* infections.

Glossina m. centralis were membrane-fed on fresh heparinised blood from Boran cow, black rhino, and goat after mixing with *T. congolense* Ng5, La3, IL 3900 and K60/1. Flies were fed on a single infective blood feed (Day 0) followed by feeds on Days 3, 6 and 10 on non-infected rabbits. They were then dissected on Days 3, 5, 10 and on Day 21 to

study the pattern of midgut infection in the flies. Initially, Kilifi (K60/1), Savannah (Ng5 and La3) and Riverine/Forest (IL 3900) were used in the investigations. However, after 6 sub-passages IL 3900 could not be detected in mice. Attempts to boost IL 3900 infections in mice by immunosuppressing with cyclophosphamide were unfruitful. As a result, only Kilifi and Savannah *T. congolense* were used in subsequent investigations.

D. Estimation of bloodmeals.

Estimation of the bloodmeal ingested by tsetse at the time of infection was achieved by weighing tsetse flies individually in small plastic tubes covered on one end with a mosquito netting (PLATE 2). Then, an estimate of the bloodmeal engorged by a fly was obtained by subtracting the weight of the fly before blood feeding (PLATE 3) from that after membrane feeding on blood from different species of animals (PLATE 4). The fly weights were recorded in milligrams (mg).

PLATE 2 Transparent plastic tubes used for holding tsetse singly during transmission of *T. congolense* from flies to mice. The top side of the tubes is covered with netting material.

PLATE 3 *Glossina morsitans centralis* **before a bloodmeal**.

5.3 RESULTS

A. Host effects on midgut infections.

Goat and zebra blood with infection rates of about 46% and 36% respectively, consist... ...fection rates than eland, ...from 5% to 22% (Fig. 6) ... ...r were significant for Ng5 ... ...also significant for K60/1 ... ...009). Goat blood in two ... ...d on two eland bloods ...

B. Eff...

... ...e (GlcNAc) in a goat blo... ...ntly increased gut infections of Ng5 ($F = 12.65$, $P = 0.006$) (Table 10, Appendix 4) and K60/1 ($F = 16.60$, $P = 0.001$) (Table 11, Appendix 5) over control flies fed on blood without chemical additives. However, Chol alone or in combination with GlcNAc did not increase infection rates of the two genotypes in tsetse infected with Boran cow blood ($P > 0.05$ for each genotype, Tables 10 and 11).

PLATE 4 *Glossina morsitans centralis* **5 minutes after a bloodmeal.**

Savannah, Kilifi and Riverine/Forest *T. congolense* showed similar patterns of midgut infection in *G. m. centralis* during the first 10 days. Infection rates peaked on Day

5.3   RESULTS

A. Host effects on midgut infections.

Goat and zebra blood with infection rates of about 46% and 36% respectively, consistently supported higher *T. congolense* (Ng5 and K60/1) midgut infection rates than eland, pig, donkey and Thomson's gazelle whose infection rates ranged from 5% to 22% (Fig. 6). Differences in infection rates for the different blood treatments were significant for Ng5 infection rates (N = 520, X^2 = 32.98, df = 5, P = 0.000) and also significant for K60/1 *T. congolense* infection rates (N = 935, X^2 = 71.17, df = 5, P = 0.000). Goat blood in two feeds significantly increased gut infection rates over flies fed on two eland bloodmeals (F = 4.97, df = 7, P = 0.034) (Table 9; Appendix 3) .

B. Effect of GlcNAc and Chol on infections.

Cholesterol (Chol) in combination with N-acetyl-D-glucosamine (GlcNAc) in a goat blood diet, fed to *G. m. centralis* at the time of infection significantly increased gut infections of Ng5 (F = 12.65, P = 0.006) (Table 10, Appendix 4) and K60/1 (F = 16.60, P = 0.001) (Table 11, Appendix 5) over control flies fed on blood without chemical additives. However, Chol alone or in combination with GlcNAc did not increase infection rates of the two genotypes in tsetse infected with Boran cow blood (P > 0.05 for each genotype, Tables 10 and 11).

C. Patterns of *Trypanosma congolense* infections.

Savannah, Kilifi and Riverine/Forest *T. congolense* showed similar patterns of midgut infection in *G. m. centralis* during the first 10 days. Infection rates peaked on Day

3 but decreased by Day 5 to reach a constant level by Day 10. This trend was observed in flies fed on goat, Boran and rhino blood (Figs. 7, 8 and 9).

Infection rates by genotypes

Tsetse dissected three days after the infective feed often showed higher K60/1 than Ng5 *T. congolense* infection rates (Figs. 7, 8 and 9). Compare 90.0% (K60/1) with 67.0% (Ng5) in females infected with rhino blood (N = 200, X^2 = 16.92, df = 1, P = 0.000) (Fig. 9). Also, compare, 86.0% (K60/1) with 40.0% (Ng5) in males infected with cow blood (N = 151, X^2 = 28, df = 1, P = 0.000). K60/1 infection rates (86.0%) were higher than Ng5 infection rates (48.0%) in males infected with goat blood (N = 176, X^2 = 25.63, df = 1, P = 0.000). K60/1 infection rates were also higher (86.0%) than IL 3900 infection rates (35.0%) in males infected with goat blood, on the 3rd day of infection (N = 340, df = 3, F = 3.73, P = 0.045) (Fig. 7).

Five days after the infective bloodmeal, no particular genotype-related trend in infection rates was noted. However, 10 days after the infective feed, infection rates of Ng5 were generally higher than infection rates of K60/1. Higher Ng5 infection rates over K60/1 were observed in tsetse infected with goat blood (40.9% *vs.* 28.0%, N = 647, df = 1, X^2 = 11.47, P = 0.001) (Fig. 7). Similar observations were noted in tsetse infected with cow blood (20.4% *vs.* 10.8%, N = 346, X^2 = 5.02, df = 1, P = 0.025) (Fig. 8).

Infection rates by fly sex.

Five days after the infective feed, differences in infection rates between sexes were generally not significant in tsetse infected with goat, Boran and rhino blood (P > 0.05 for all treatments, Figs. 8 and 9). However, after 10 days of infection, significant sex-related differences in infection rates were observed in tsetse infected with K60/1 *T. congolense* in

rhino blood (21.6% males *vs.* 6.0% females; Fisher's Exact test' N = 120, P = 0.020 - Fig, 9).

Day 10 *vs.* day 21 midgut infections.

Infection rates in flies dissected on Day 10 were not significantly different from those dissected on Day 21 in tsetse infected with *T. congolense* K60/1 in pig, zebra, goat and eland blood (All treatments, $P > 0.05$, Table 12).

D. Estimation of Bloodmeals.

Bloodmeal sizes ingested by *G. m. centralis* fed on blood of various hosts ranged from 22.3 mg to 27.5 mg. No significant differences in meal sizes were observed between blood treatments (Table 13).

Table 9

Trypanosoma congolense **(K60/1) midgut infection rates in** *G. m. centralis* **membrane fed on double goat (2 Goat) or double eland (2 Eland) or eland followed by goat (Eland:Goat) or goat followed by eland blood (Goat:Eland), dissected 10 days after infection.**

Feeding pattern	% of tsetse infected (Mean ± SE)
2 goat	21.90 ± 0.67 a
Goat:Eland	21.77 ± 8.00 a
Eland:Goat	13.89 ± 4.90 a b
2 Eland	7.74 ± 1.04 b

N = 200 for each treatment.

Means with different letters between rows are significantly different at $P < 0.05$;

SE: Standard error.
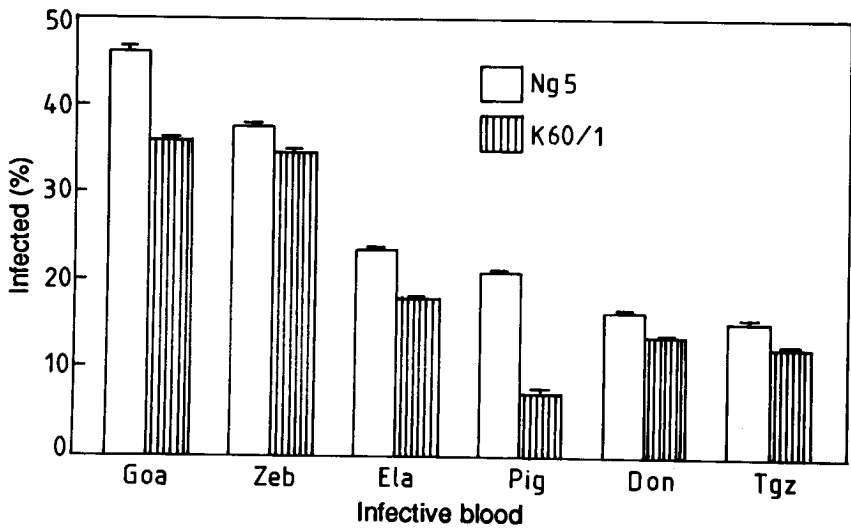
Figure 6   Effect of goat (Goa), zebra (Zeb), eland (Ela), pig, donkey (Don) and Thomson's gazelle (Tgz) blood on midgut infection rates of Ng5 and K60/1 in male and female *G. m. centralis* dissected 10 days after infection.

(N = 1500, equal numbers per treatment).

Table 10

Effect of GlcNAc and Chol on midgut infection rates(%) of *T. congolense* **Ng5 in** *G. m. centralis,* **fed on goat and Boran cow blood and dissected 10 days after infection.**

Treatment	Number of tsetse dissected	% of tsetse infected (Mean ± SE)
Cow blood		
GlcNAc + Chol	222	28.08 ± 7.60 a
Chol	86	20.05 ± 2.00 a
Control	178	16.09 ± 4.00 a
Goat blood		
GlcNAc + Chol	203	47.00 ± 6.00 a
Control	140	16.18 ± 5.60 b

Means followed by different letters are different at $P < 0.05$;

GlcNAc: N-acetyl-D-glucosamine; **Chol:** cholesterol; **SE:** Standard error.

Table 11

Effect of GlcNAc and Chol on midgut infection rates(%) of *T. congolense* **K60/1 in** *G. m. centralis***, fed on goat and Boran cow blood and dissected 10 days after infection.**

Treatment	Number of tsetse dissected	Number tsetse infected (Mean ± SE)
Cow blood		
GlcNAc + Chol	145	23.50 ± 5.80 a
Chol	155	9.30 ± 2.85 a
Control	167	12.90 ± 3.69 a
Goat blood		
GlcNAc + Chol	225	44.90 ± 7.00 a
Control	229	25.60 ± 3.00 b

Means followed by different letters are significantly different at $P < 0.05$;

GlcNAc: N-acetyl-D-glucosamine; **Chol**: cholesterol; **SE**: Standard error.
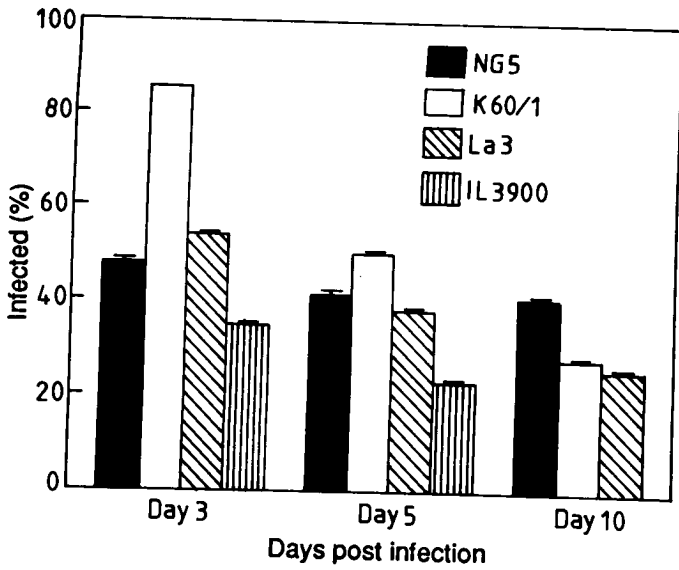
Figure 7   **Patterns of Savannah (Ng5, La3), Kilifi (K60/1) and Riverine/Forest (IL 3900)** *T. congolense* **infection rates(%) in male** *G. m. centralis* **membrane fed once on goat blood and dissected on days 3, 5 and 10 after infection.**
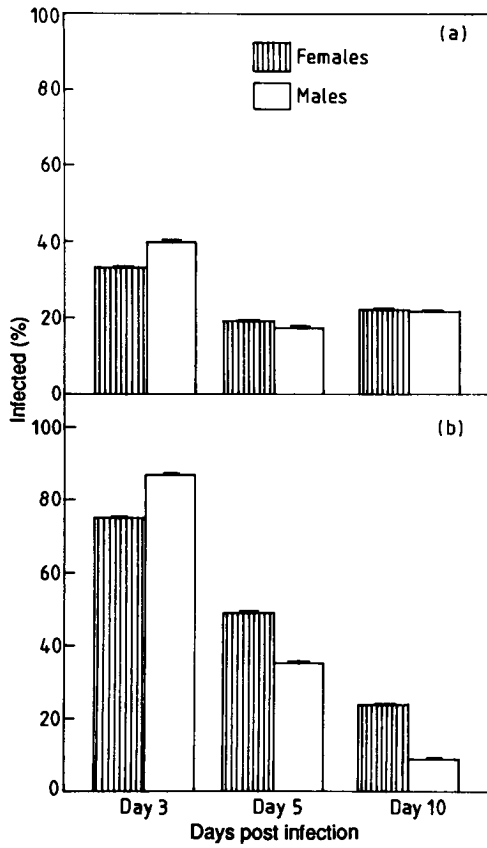
Figure 8 Patterns of (a) Ng5 and (b) K60/1 *T. congolense* infection rates(%) in male and female *G. m. centralis* infected with Boran cow blood and dissected on days 3, 5 and 10 after the infective feed.
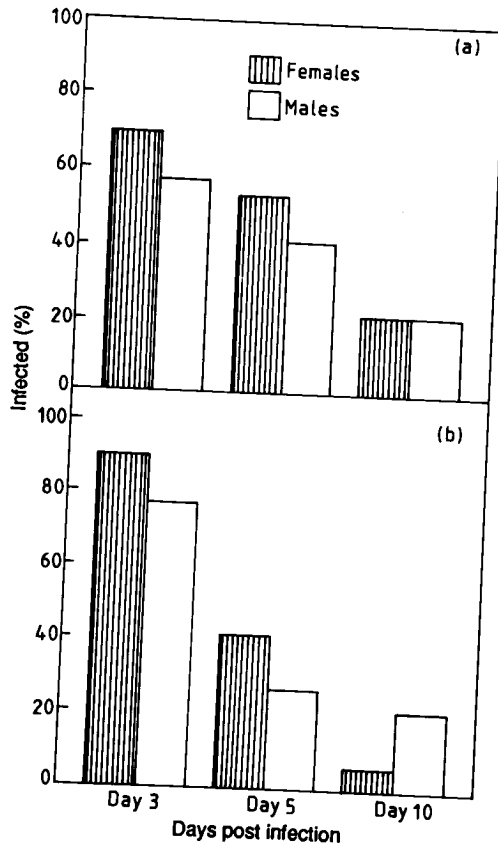
Figure 9   Patterns of (a) Ng5 and (b) K60/1 *T. congolense* infection rates(%) in male and female *G. m. centralis* infected with black rhino blood and dissected on days 3, 5 and 10 after the infective feed.

Table 12

Midgut infection rates(%) of *T. congolense* **in** *G. m. centralis* **fed on pig, zebra, eland and goat blood, dissected 10 and 21 days after infection.**

Trial	Blood	Day tsetse dissected	N	% infected (Mean $\pm$ SE)	*P*
1	Pig	10	89	17.4 $\pm$ 8.83	
1	Pig	21	88	28.4 $\pm$ 1.14	0.34
2	Zebra	10	81	27.0 $\pm$ 1.47	
2	Zebra	21	84	29.0 $\pm$ 5.00	0.75
3	Eland	10	85	23.6 $\pm$ 2.63	
3	Eland	21	86	22.0 $\pm$ 1.16	0.67
4	Goat	10	78	33.0 $\pm$ 6.00	
4	Goat	21	74	52.0 $\pm$ 3.22	0.15

N = Number of tsetse dissected;   **SE:** Standard error;   *P*: probability.

Table 13

Bloodmeal sizes of *G. m. centralis* **fed on eland, zebra, goat, Boran cow, pig and black rhino blood at the first infective feed.**

Blood	Bloodmeal (mg) Mean ± SE
Eland	27.50 ± 3.00 a
Zebra	26.50 ± 3.07 a
Goat	26.20 ± 0.91 a
Boran	25.20 ± 0.93 a
Pig	25.00 ± 1.79 a
Rhino	22.30 ± 1.58 a

N = 2840, equal numbers per treatment; Means with the same letters between rows are not significantly different at $P < 0.05$; mg: milligrams; SE: Standard error.

5.4 DISCUSSION

This study shows that goat blood supports the development of Kilifi and Savannah *T. congolense* midgut infections better than cattle, pig, donkey, rhino, Thomson's gazelle and eland blood. This unique property of goat blood enhancing midgut infection rates was also reported by Mihok *et al.* (1993) and Olubayo *et al.* (1994) using Savannah *T. congolense* and *T. b. brucei*.

The mechanism by which goat blood facilitates midgut infection rates in tsetse remains unknown. However, useful clues may lie in the carbohydrate molecules on the red blood cell (Olubayo *et al.*, 1994). Ingram and Molyneux (1988) reported that human ABO(H) type RBCs possess carbohydrate molecules that specifically bind to lectins from tsetse gut and haemolymph. Midgut infection rates supported by various hosts' blood suggested that the facilitation effect of goat blood may not be a result of the small size of goat RBCs (Mihok *et al.*, 1994a; Mihok *et al.*, 1995). It is possible that exposed carbohydrate moieties on goat red blood cells, unlike other host RBCs, result in a better combination with lectins thereby blocking the latter from killing trypanosomes. This would account for the increased infection rates in tsetse infected with goat blood.

Experiments involving feeding tsetse on reconstituted, infective blood mixtures support the idea that RBCs play a role in facilitating trypanosome infections. A significant increase in infections was achieved by feeding tsetse on a diet containing a high proportion of RBCs than when feeding tsetse on whole goat blood (Mihok *et al.*, 1994a). Also, a diet of cow RBCs suspended in saline resulted in over 90% gut infection of *T. congolense* in *G. m. morsitans* compared with 60% in control flies (Maudlin *et al.*, 1984). Moloo (1981) showed that the type of animal species used for maintaining tsetse after the infective

bloodmeal influences the vector's subsequent infection rates with pathogenic trypanosomes. Rabbits as maintenance hosts supported higher rates of *T. congolense*,

T. b. brucei, and *T. vivax* infections than laboratory rodents. Cheeseman and Gooding (1985) characterised several proteolytic enzymes including trypsin, carbo-peptidases and amino-peptidases from adults and larvae of *G. m. morsitans*, *G. m. submorsitans* and *G. m. centralis*. Imbuga *et al.* (1992) and Osir *et al.* (1993) demonstrated that D(+)-glucosamine, the sugar that inhibits midgut lectins also specifically inhibited midgut trypsin, suggesting a possible role of digestive proteases in gut establishment of trypanosomes. Mihok *et al.* (1994b) and Mihok *et al.* (1995) using *T. congolense*,

T. simiae and *T. b. brucei*, measured midgut protease activity of *G. m. morsitans* and *G. m. centralis* after an infective feed on blood from several wildlife and livestock species. Protease activity was generally unrelated to infection rates despite large differences in infection rates between the two tsetse species. Also, host blood that repeatedly increased infection rates (goat) stimulated similar enzyme activity to host blood that reduced infection rates (buffalo). Nevertheless, addition of D(+)-glucosamine to parasitaemic blood resulted in a three-fold reduction in protease activity and a large increase in infection rates. This effect did not occur when trypanosomes or glucosamine were added alone to the bloodmeal. They suggested that the effect on enzyme activity might be through a pH change by ammonia resulting from glucosamine metabolism.

In the present investigation *G. m. centralis* fed on zebra and goat blood consistently developed significantly higher rates of *T. congolense* infections than flies fed on cattle, donkey, pig, Thomson's gazelle and eland infective blood despite taking similar sizes of bloodmeals. Higher infection rates in zebra-fed tsetse are of particular interest. Zebra is not a favoured host of *Glossina* species as shown by bloodmeal analysis (Weitz, 1963). Also, crude surveys show low prevalence rates of trypanosomes in zebra (Dillman and Townsend, 1979) but the physiological basis for this remains to be elucidated. Zebras

and horses are classified under the same family Equidaes, whereas goats, cattle and buffaloes are grouped in the Bovidae family. Similar infection rates of *T. congolense* in tsetse infected with blood from different families is puzzling. An investigation to determine the factors in zebra and goat blood which are similar is quite desirable. Savannah, Kilifi and Forest *T. congolense* types showed similar patterns of midgut infection rates in *G. m. centralis* during the first 10 days of infection; infection rates were highest on Day 3 but decreased between Days 5 and 10. Infection rates in flies dissected on Day 10 were similar to those of flies dissected on day 21. Olubayo *et al.* (1994) observed a similar pattern of infection rates in tsetse infected with a Savannah *T. congolense* type and *T. b. brucei*. These observations suggest that critical events for *T. congolense* establishment occur early in the parasite's development in tsetse.

The peritrophic membrane was reported to be the main barrier to trypanosome establishment in older (non-teneral) tsetse (Wijers, 1958; Willet (1966). However, Ellis and Evans (1977) observed that *T. b. rhodesiense* could penetrate the peritrophic membrane into the peritrophic space of *G. m. morsitans*, located between the membrane and the gut wall. This observation was confirmed by Evans *et al.* (1979), using *T. congolense* infections in the same tsetse species. Moreover, Mshelbwala (1972) observed trypanosomes in tsetse haemolymph. Recently, Welburn and Maudlin (1992) demonstrated that tsetse fed on D(+)-glucosamine at the second, third or even seventh subsequent feed, developed infection rates comparable with those of tenerals. This disproved Willet (1966)'s assertion that the peritrophic membrane acts as a mechanical barrier preventing older flies from becoming infected.

If the peritrophic membrane is not the main barrier to trypanosome development in tsetse, then, the question arises: what limits trypanosome development?-is it an immune system similar to that in mammalian hosts? There is a paucity of information regarding adult insect immune systems and even less on tsetse (Maudlin, 1991; Kaaya, 1989). Lackie

(1988) reviewed vector immunity in general, whereas Kaaya (1989) reviewed immunity in tsetse. It is clear that insects lack lymphocytes, the cornerstone of mammalian immunity and also lack immunoglobulins and complement (Boman and Hultmark, 1987). However, cellular and humoral immunity have been identified in insects (Lackie, 1988; Kaaya, 1989) but evidence of cellular responses against trypanosomes in tsetse is lacking.

Escherichia coli injected into the haemocoel of *G. m. morsitans* were quickly phagocytised by haemocytes, but phagocytosis of *T. b. brucei* was not observed, suggesting no involvement of cellular immunity in tsetse (Kaaya *et al.*, 1986). However, *T. b. brucei* provoke humoral responses when injected into tsetse haemocoel. Humoral responses against trypanosomes were also demonstrated by Croft *et al.* (1982) who reported a factor in *G. m. morsitans* haemolymph which reduced the motility of *T. b. brucei* culture forms. The haemolymph factor was an agglutinin with a distinct sugar specificity - a lectin. Ibrahim *et al.* (1984) observed agglutination of *T. b. brucei* by midgut extracts from *G. austeni*. Involvement of trypanolysins and trypanoagglutinins in killing trypanosomes has been confirmed by Stiles *et al.* (1990). There are various possible factors that limit trypanosome development. For this reason, the factor(s) responsible for reductions of midgut infections between Days 3 and 10 must involve a complex interaction between proteases, lysins and agglutinins. Metabolic byproducts of chitin (e.g. ammonia) as discussed, might indirectly influence the activity of protein digestive enzymes and probably influence parasite establishment. The role of lipids in this trypanosomatid-gut interaction should not be ruled out since addition of cholesterol in two tsetse bloodmeals significantly increased midgut infections over control flies (Chapter 4).

Higher midgut infections of Savannah compared to Kilifi and Riverine/Forest *T. congolense*, in goat-fed flies may have been associated with differences in numbers of lectin binding receptors on the surface of trypanosomes which Maudlin and Welburn (1988) reported to vary between trypanosome stocks. Differences in trypanosome

infectivity to tsetse reported here confirm the genetic variation within *T. congolense* shown at the molecular level (Majiwa *et al.*, 1985; Gashumba, 1990; Garside *et al.*, 1994). Diversity within different species of parasites has adaptive value: it increases chances of parasite survival in hosts. An understanding of trypanosome transmission from tsetse to mammalian hosts is essential in formulating effective control strategies. The next chapter reports on this subject.

CHAPTER 6

HOST INFLUENCE ON MATURATION AND TRANSMISSION OF *Trypanosoma congolense*

6.1 INTRODUCTION

Trypanosoma congolense ingested in a bloodmeal undergoes several morphological and biochemical developmental stages in tsetse (Molyneux and Ashford, 1983; Vickerman, 1985; Stebeck and Pearson, 1994). During this development some parasites die, whereas others establish as procyclics and eventually migrate to the labrum to transform into epimastigotes. Finally, the epimastigotes enter the hypopharynx where they transform into the metacyclic stage infective to vertebrate hosts (Dipeolu, 1975; Evans *et al.*, 1979).

The development of metacyclic trypanosomes in tsetse is essential for their successful transmission to the mammalian host in which they cause disease. Consequently, the subject has stimulated a lot of interest (Buxton, 1955; Jordan, 1974; 1976; Molyneux, 1986; Molyneux and Stiles, 1991; Ingram and Molyneux, 1991; Maudlin, 1991; Maudlin and Welburn, 1994). Recently, the role of the insect immune system in influencing the development of filarial and malarial parasites to the infective stage has also been reported (Perrone *et al.*, 1986; Ham *et al.*, 1991). Despite these advances, the mechanisms by which the insect immune system affects parasite maturation are unknown. However, Welburn and Maudlin (1990) have demonstrated that midgut lectins may mediate maturation of *T. b. brucei* in tsetse.

There are conflicting reports on the influence of fly sex on maturation of *T. congolense*. Maudlin *et al.* (1991) analysed data from several sources and proposed that maturation of *T. congolense* in *Glossina* is independent of fly sex. However, Distelmans *et al.* (1982) and Moloo *et al.* (1992a) reported higher maturation in male than female *G. palpalis palpalis* and *G. m. centralis*. Information on maturation rates of Savannah, Kilifi and Riverine *T. congolense* sub-types in *Glossina* species is limited. There are few studies (Maudlin *et al.*, 1984; Mihok *et al.*, 1991; Mihok *et al.*, 1993; Olubayo *et al.*, 1994) on the influence of host blood on *T. congolense* infections in the vector and yet such information may lead to a better control of African trypanosomiasis. It is known that infection rates in hosts vary between species and stocks of trypanosomes (Buxton, 1955; Dukes *et al.*, 1989; Maudlin and Welburn, 1988; Maudlin and Welburn, 1994). Whether variation in infectivity to *Glossina* and mammalian hosts occurs between Kilifi, Savannah and Forest *T. congolense* variants is unknown (Gashumba, 1990).

The study reported in this Chapter was therefore undertaken with the general objectives of (1) investigating the effect on maturation rates of *T. congolense* types when mixed with blood of various livestock and wildlife species (2) determining the influence of host blood on transmission rates to mice and (3) establishing the base-line information on the role of various strains *T. congolense* in livestock morbidity and mortality.

6.2 MATERIALS AND METHODS

A. Maturation of *Trypanosoma congolense* in *Glossina m. centralis.*

i. Host influence on hypopharynx infections.

Experiments were designed to investigate the influence of host blood on labrum and hypopharynx infections. The proportion of midgut infections that matured into hypopharynx infections was referred to as the transmission index (PLATE 5). The transmission index (TI) was expressed as:

Hypopharynx infections / Midgut trypanosome infections

100

Teneral male and female *G. m. centralis* in groups of 50 were membrane-fed on fresh blood from a goat, pig, donkey, zebra, eland and Thomson's gazelle after mixing with *T. congolense* Ng5 and K60/1. Estimation of trypanosomes in the infective bloodmeal was done, using a haemacytometer. Thirty days after infection, tsetse were dissected for presence of trypanosomes using procedures described in chapter 3 above.

ii. Host influence on metacyclic loads.

Teneral *G. m. centralis* in groups of 50 were membrane-fed on fresh heparinised blood from a zebra, Thomson's gazelle, eland, goat, pig and donkey mixed with *T. congolense*. Labral and hypopharynx infections were examined for presence of trypanosomes as described above (Chapter 3). For each blood treatment, tsetse with heavy 'hypopharynx' infections were recorded. For each fly, infections with 40 or more

metacyclic trypanosomes per hypopharynx were considered as "heavy". The proportion of tsetse showing 'Heavy' metacyclics was expressed as a percentage over the total infected in the hypopharynx.

iii Role of genotype on maturation rates.

Two groups of teneral male *G. m. centralis* were each infected with Ng5 and K60/1 *T. congolense* in goat, donkey, Thomson's gazelle and zebra blood and dissected 30 days later for labral and hypopharynx infections.

iv. Influence of GlcNAc and GlcN on maturation.

To compare the effect on metacyclic numbers of feeding tsetse on N-acetyl-D-glucosamine (GlcNAc) and D(+)-glucosamine (GlcN) during the infective bloodmeal, groups of one day old male and female flies were fed on fresh goat or eland blood mixed with GlcNAc and GlcN to a final concentration of 0.05 M. Control flies were fed on blood mixed with trypanosomes alone. Tsetse were maintained on healthy rabbits and dissected 30 days after infection.

The effect of D(+)-glucosamine was compared in three bloodmeals on metacyclic infections. Groups of 50 teneral male and female *G. m. centralis* were infected on Day 0 with goat blood mixed with bloodstream form trypanosomes mixed with GlcN 0.05 M. They were fed again on the goat blood/GlcN mixture (without trypanosomes) on Days 2 and 5. Control flies were similarly fed on blood with no glucosamine. Tsetse were dissected after 30 days of infection as described in Chapter 3.
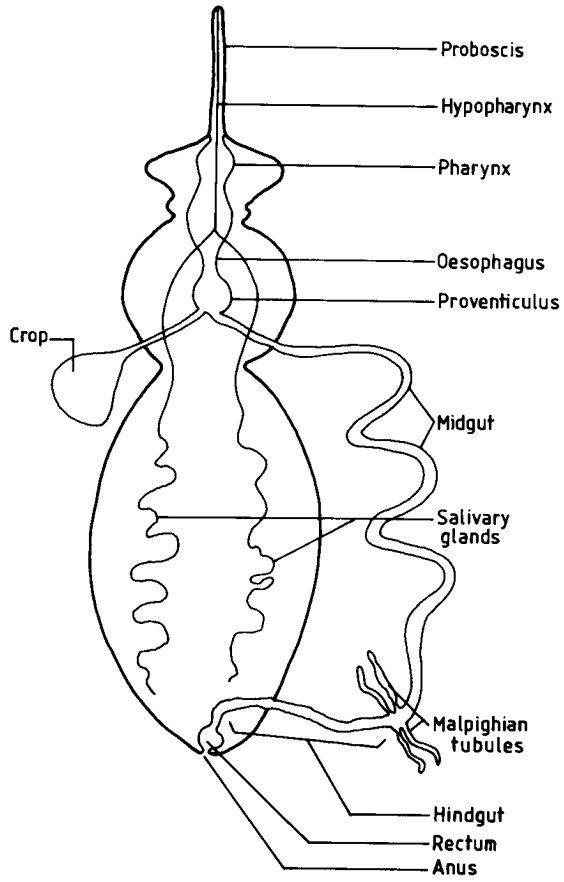
PLATE 5   **Diagram of an adult tsetse fly showing the hypopharynx and the alimentary canal.**

B. Transmission from tsetse to mice.

i. Host blood influence.

Teneral male and female *G. m. centralis* in groups of 50 were membrane fed on goat, donkey, eland and zebra blood after mixing with bloodstream form *T. congolense* Ng5 and K60/1. Tsetse were maintained for 30 days to allow the trypanosomes to mature. To identify infected tsetse for use in transmission experiments, the 'salivation' method described by Burtt (1946) was used. However, in several attempts, trypanosomes were not detected in saliva extruded by tsetse shown by dissection, to harbour numerous metacyclics in their hypopharynx. Due to this reason, the following alternative procedure was adopted: Tsetse were isolated into single fly tubes (PLATE 2) and fed on mice after two days of starvation. Fed flies were given a number corresponding to that of the mouse on which they fed, and were dissected two days later to confirm infections. Flies which did not feed were excluded from the experiment. The mice on which tsetse (confirmed to harbour hypopharynx infections) fed were maintained in plastic cages, and kept for examination. Tail blood from each of these mice was collected and examined at 400x magnification daily for one month. The transmission rates were thus determined.

ii. Transmission rates by genotype and sex of tsetse.

Three groups of teneral *G. m. centralis* males and females were each infected with K60/1, La3 and Ng5 *T. congolense* genotypes in goat blood. Thirty days after infection the flies were isolated into single fly tubes and fed once on mice in order to assess transmission rates to the latter. Inoculated mice were placed into separate cages according to the genotype of the infecting flies and examined for trypanosome infections as described earlier .

Three groups of teneral male and female *G. m. centralis* were fed on blood from a goat, eland and donkey after mixing with *T. congolense* K60/1 and Ng5. Thirty days after infection, the flies were isolated into single fly tubes and fed once on mice in order to assess transmission rates to the latter using the procedure described in this chapter on page 74, section 6.2 A. (i). Inoculated mice were placed into separate cages according to the sex of the fly used for transmission and examined for trypanosome infections at 400x magnification. The experiment on influence of fly sex on transmission rates was replicated six times.

6.3 RESULTS

A. Maturation rates of *Trypanosoma congolense* **in** *Glossina m. centralis.*

(i) Host influence on hypopharynx infection rates and metacyclic loads.

Zebra and goat blood supported the highest labral infection rates as compared to pig, eland, donkey and Thomson's gazelle blood (Table 14) (Appendix 6). Zebra blood alone supported the highest hypopharynx infection rates and metacyclic numbers (Table 15) (Fig. 10) (Appendix 7). There was no interaction between blood type and fly sex (blood x Sex) or between *T. congolense* genotype and fly sex (genotype x sex) for labral ($P > 0.05$, Appendix 6) and hypopharynx ($P > 0.05$, Appendix 7) infections. The transmission index expressed as a percentage of hypopharynx infection over midgut infection was generally high in most blood treatments, ranging from about 58.0% for tsetse infected with eland to 96.0% for tsetse infected with zebra blood (Table 16). Male *G. m. centralis* infected with zebra and eland blood matured more midgut infections of *T. congolense* than females (Table 16). However, the increases in mature infection rates supported by male compared to female flies infected with goat and pig blood were not significant ($P > 0.05$ for both treatments) (Table 16).

ii. Influence of genotype, GlcNAc and GlcN on maturation rates.

Except for tsetse infected with pig blood, Ng5 and K60/1 *T. congolense* infection rates were generally similar in *G. m. centralis* infected with, goat, Thomson's gazelle, donkey and zebra blood (Tables 17 & 18; Appendix 8).

GlcNAc in a single bloodmeal significantly increased the proportion of male *G. m. centralis* developing 'heavy' hypopharynx infection compared to controls (Table 19). Similarly, GlcN in a single bloodmeal significantly increased the proportion of female flies developing "heavy" hypopharynx infection compared to controls (Table 19). In three goat bloodmeals, GlcN significantly increased the transmission index of female *G. m. centralis* over control flies (Table 20).

B. Transmission of *T. congolense* from tsetse to mice.

Transmission rates of *T. congolense* into mice from tsetse fed on blood of different hosts were generally similar (*P*=0.05) (Table 21; Appendices 9 and 10).

Comparisons of transmission rates between Ng5, K60/1 and La3 are shown in Table 22 and Appendix 11. Transmission rates between genotypes did not differ significantly from each other at *P* = 0.05.

As regards the effect of fly sex on transmission rates, male *G. m. centralis* were significantly better vectors for *T. congolense* than their female counterparts (Table 23; Appendix 12). Transmission rates of flies infected with blood of different hosts were generally similar, as earlier reported in this section.
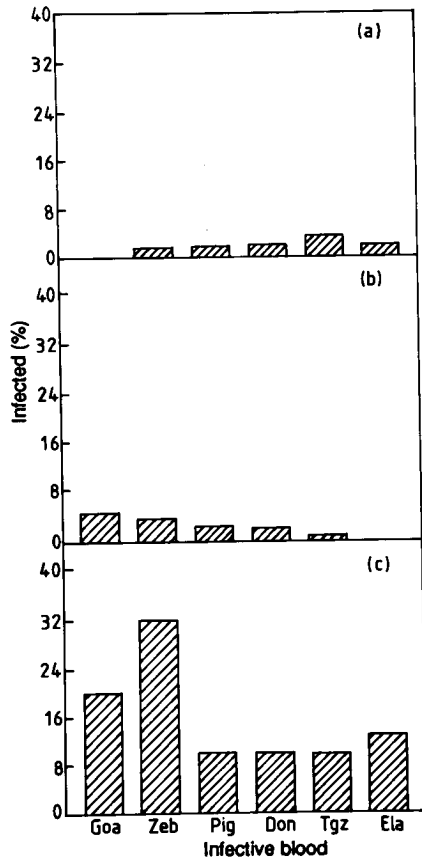
Figure 10　　**Influence of host blood on the development of metacyclic infection (Means) in the hypopharynx of** *G. m. centralis*; **(a) Light (b) Medium (c) Heavy metacyclic infection.**

(N = 223 for each treatment. Heavy: 40 or more metacyclics per hypopharynx).

Table 14

Influence of host blood on labrum infection rates of *T. congolense* ***(Mean ± SE) in**

G. m. centralis.

Blood	% of tsetse infected
Zebra	33.63 ± 3.21 a
Goat	33.45 ± 5.74 a
Pig	18.17 ± 2.09 b
Eland	15.82 ± 1.90 b
Donkey	13.13 ± 1.30 b
Thomson's gazelle	12.62 ± 2.30 b

$N = 200$ for each treatment; Means followed by different letters between rows are significantly different at $P < 0.05$; *T. congolense**: Ng5 and K60/1 were pooled since no significant difference in infection rate was found between them at $P = 0.05$; **SE:** Standard error.

Table 15

Influence of host blood on hypopharynx infection rates of *T. congolense*[*] **(Mean ±**
SE) in *G. m. centralis.*

Blood	% of tsetse infected
Zebra	32.48 ± 3.30 a
Goat	25.75 ± 6.49 b a
Eland	15.49 ± 1.82 b
Pig	15.37 ± 1.78 b c
Donkey	11.96 ± 0.82 b c
Thomson's gazelle	10.56 ± 2.63 c

N = 200 for each treatment; Means followed by different letters between rows are
significantly different at *P* < 0.05; *T. congolense*[*] = Ng5 and K60/1.

Table 16

Influence of fly sex on transmission index (TI)* of *T. congolense* **in** *G. m. centralis.*

Clone	Blood	Sex	TI (%)	X^2	P
K60/1	Goat	M	76.19		
K60/1	Goat	F	61.29	1.26	0.26
K60/1	Eland	M	88.24		
K60/1	Eland	F	57.69	7.33	0.007
K60/1	Zebra	M	93.00		
K60/1	Zebra	F	88.89	0.45	0.50
Ng5	Pig	M	86.67		
Ng5	Pig	F	81.25	0.17	0.68
Ng5	Zebra	M	96.67		
Ng5	Zebra	F	72.34	7.28	0.007

N = 100 tsetse were dissected for each treatment; **F:** Female; **M:** Male; X^2: Chi-square;

P. Probability. **TI*** = Hypopharynx infections / Midgut trypanosome infections.

100

Hypopharynx infections of *T. congolense* **in male** *G. m. centralis* **by genotype.**

Trial	Clone	Blood	Diss.	% Inf.	X^2	P
1	Ng5	Goat	74	35.0		
1	K60/1	Goat	60	35.0	0.000	0.987
2	Ng5	T. gaz	92	14.0		
2	K60/1	T. gaz	124	9.7	1.020	0.312
3	Ng5	Donkey	86	12.8		
3	K60/1	Donkey	89	11.2	0.100	0.752
4	Ng5	Zebra	38	34.2		
4	K60/1	Zebra	39	43.6	0.712	0.399
5	Ng5	pig	72	18.0		
5	K60/1	pig	93	14.0	80.76	0.000

T. gaz: Thomson's gazelle;   **inf.:** infected;   **Diss.:** dissected; X^2: Chi-square Test;

P: Probability.

Table 18

**Development of 'heavy' metacyclic infections in the hypopharynx of *G. m. centrali*
infected with goat, donkey, Thomson's gazelle and zebra blood by genotype.**

Clone	N	% of tsetse infected (Mean ± SE)
Ng5	333	21.90 ± 3.00 a
K60/1	598	20.00 ± 2.50 a

N = Number of tsetse dissected; Means followed by the same letter between rows are not
significantly different at $P < 0.05$; SE: Standard error.

Table 19

Effect of GlcNAc and GlcN on development of heavy* metacyclic infections in the hypopharynx of *G. m. centralis* **infected with** *T. congolense* K60/1.

Trial	Trt	Bood.	Sex	Diss.	Inf. %	X^2	P
1	GlcN	Goat	M	153	52.1		
1	Control	Goat	M	113	68.0	0.037	0.848
2	GlcNAc	Goat	M	113	90.5		
2	Control	Goat	M	113	68.0	5.39	0.020
3	GlcN	Eland	M	95	65.0		
3	Control	Eland	M	112	77.8	0.84	0.360
4	GlcN	Goat	F	53	76.7		
4	Control	Goat	F	106	41.0	6.86	0.009
5	GlcNAc	Goat	F	34	62.5		
5	Control	Goat	F	106	41.9	1.10	0.295
6	GlcNAc	Eland	F	67	53.3		
6	Control	Eland	F	68	50.0	1.36	0.243

GlcNAc: N-acetyl-D-glucosamine; **GlcN:** D(+)-glucosamine; **Heavy*:** 40 or more metacyclics per hypopharynx; **Diss.:** Dissected; **Trt.:** Treatment; **Inf.:** Infected; X^2: Chi-square; *P*: Probability.

Table 20

Influence of GlcN in three bloodmeals on transmission index (TI) in *G. m. centralis* **infected with** *T. congolense* K60/1 **in goat blood.**

Sex	Trt.	TI**	*P*
Female Female	**3x GlcN*** Control	87.88 61.29	 0.014
Male Male	**3x GlcN** Control	69.81 73.00	 0.73

N = 130 tsetse dissected per treatment; **3 GlcN***: Three bloodmeals on D(+)-glucosamine;

Trt.: treatment;

TI: Transmission index = Hypopharynx/Midgut trypanosome infections.

100

Table 21

Influence of host blood fed to tsetse during the first bloodmeal on transmission rates of *T. congolense* **to mice by** *G. m. centralis.*

Blood	Number of mice bitten[*]	% of Mice infected (Mean $\pm$ SE)
Trial 1		
Zebra	22	37.5 $\pm$ 0.80 a
Pig	20	47.0 $\pm$ 5.00 a
Donkey	17	54.5 $\pm$ 2.00 a
Trial 2		
Eland	63	47.4 $\pm$ 3.60 a
Goat	80	44.3 $\pm$ 10.50 a

Bitten[*]: Mice singly bitten by infected tsetse; Means followed by the same letter between rows are not significantly different at *P* < 0.05;   **SE:** Standard error.

Table 22

Transmission rates of *T. congolense* into mice by genotype.

Clone	Number of mice bitten	% of mice infected (Mean ± SE)
Ng5	71	42.61 ± 8.0 a
K60/1	70	30.83 ± 4.0 a
La3	56	30.53 ± 12.0 a

Means followed by the same letter between rows are not significantly different at

P < 0.05;   SE: Standard error.

6.4 DISCUSSION

Zebra blood facilitates maturation of *T. congolense* in *G. m. centralis* much better than other species of wildlife and livestock hosts. This suggests presence of some factors in this animal that are absent in other animals including the donkey which is closely related to zebra. Such factors probably include, among other things, interactions between some components of the fly midgut, blood components and the trypanosome. According to Maudlin and Welburn (1987, 1988), maturation of *T. congolense* in tsetse depends on a signal from midgut lectins. The latter are involved in procyclic maturation of trypanosomes in tsetse as revealed by several observations. Maudlin and Welburn (1990) revealed that addition of the carbohydrate melibiose to the tsetse bloodmeal blocks maturation of both *T. congolense* and *T. b. brucei* in *G. m. morsitans*. They then attributed the observations to inhibition of the haemolymph lectins. Furthermore, continuous inhibition of midgut lectins by glucosamine has been demonstrated to block maturation (Maudlin and Welburn, 1988). Mutharia and Pearson (1987) found surface exposed N-acetyl-D-glucosamine moieties on *T. congolense* procyclics but not on *T. brucei gambiense* which matures poorly in tsetse.

If midgut lectins play a role in maturation of *T. congolense*, then higher maturation rates in tsetse fed on a zebra infective bloodmeal compared to other types of blood may be attributed to lectin stimulation. Trypanosomes in a zebra bloodmeal are probably more stimulated than those fed on blood of other animal species. This may be possible if surface carbohydrates on zebra RBCs possess a better match for tsetse midgut lectins than those of other wildlife species. Whatever the mechanism, this study suggests a species-specific blood influence on *T. congolense* maturation in *G. m. centralis*. Addition of a lectin inhibitor in three bloodmeals significantly increased the maturation rates of *T. congolense* in female flies compared to the controls. This suggests that the mechanism controlling maturation of *T. congolense* involves lectins.

Zebra blood increased *T. congolense* infections in both midgut and hypopharynx, thus suggesting that it possesses essential factors necessary for complete cyclical development of *T. congolense* in tsetse. High transmission indices of about 63.0% to 90.4% in tsetse fed on wildlife and livestock species, suggest that very little barrier to maturation existed once trypanosomes established in the tsetse gut. However, this observation may not apply to other stocks of trypanosomes.

Maudlin and Welburn (1988) observed differences in maturation rates between different stocks of *T. congolense* and concluded that maturation of *T. congolense* depended on the genotype. They attributed variations in maturation to differences in numbers of lectin binding sites on the surface of trypanosomes. However, in the present investigation no significant differences in maturation rates were observed between the various *T. congolense* genotypes.

Maudlin *et al.* (1991) analysed data from several sources and concluded that maturation of *T. congolense* in *Glossina* was independent of fly sex, whereas that of *Trypanozoon* was controlled by a sex-linked gene on the X-chromosome of male tsetse. Distelmans *et al.* (1982) reported a higher maturation rate in male than female *G. palpalis palpalis* infected with a *T. congolense* stock from Uganda. Also, Moloo *et al.* (1992a) observed that male *G. m. centralis* were more susceptible to *T. congolense* infection than females. They suggested that maturation of *T. congolense* in *Glossina* was genetically controlled.

This study shows that male *G. m. centralis* supports significantly higher maturation rates of midgut *T. congolense* than female flies when fed infected blood of wildlife and domestic animals. This is in contrast with Maudlin *et al.* (1991) but consistent with the observations of Moloo *et al.* (1992a) whose findings were based on cattle and goats as hosts. A lectin inhibitor given in three bloodmeals including the infective feed resulted in a higher maturation in female *G. m. centralis* than in males, indicating sex differences in *T.*

congolense maturation. These results have important implications on the Sterile Male Technique (SMT) of controlling tsetse flies (see Chapter 8).

There is little direct evidence for genetic control of vectorial capacity in tsetse (Gooding, 1984; 1992). No genetic mechanism has been demonstrated for trypanosome maturation but there is evidence that susceptibility to midgut infections of *T. congolense* is inherited from the female fly (Maudlin, 1982; Maudlin and Ellis, 1985). Mcdonald (1962) reported that susceptibility to filarial infection in mosquitoes was genetically controlled. It is possible that maturation of *T. congolense* in *Glossina* species is also genetically controlled, but further studies are needed to substantiate this possibility.

In the current investigation, no significant differences in transmission rates between various *T. congolense* types into mice by tsetse fed on blood of various host species were demonstrated. Although the results provide useful baseline information, it is important to perform similar studies on larger mammalian hosts. Further studies should also identify and characterise the factor(s) in host blood that are responsible for supporting cyclical development of trypanosomes in *Glossina* species for possible use in novel strategies to block disease transmissions. Furthermore, there is limited information on the influence of host blood fed to the vector on subsequent behaviour of trypanosomes in the mammalian host. Chapter 7 describes some investigations and observations on such influence.

CHAPTER 7

INFLUENCE OF HOST ON VIRULENCE OF *Trypanosoma congolense* METACYCLICS

7.1 INTRODUCTION

Vertebrate blood provides the sole source of nutriment for tsetse. Adults feed on blood only and larvae are nourished *in utero*. The bloodmeal is used for growth, reproduction and provision of energy. The balance, if any, is used for making food reserves, mainly lipids (Bursell *et al.*, 1974; Tobe and Langley, 1978).

The bloodmeal is also vital to the establishment of trypanosomes in the vector (Vickerman, 1985). However, host blood differs in its ability to support trypanosome development in tsetse. Goat blood for example, supports the development of trypanosomes in tsetse better than other types of blood when given to tsetse at the infective feed (Mihok *et al.*, 1993; Olubayo *et al.*, 1994). Since host blood supports the development of trypanosomes differently, the possibility that it similarly affects virulence of these parasites on mammals cannot be ruled out although such effects have not yet been demonstrated. However, virulence of some *Nannomonas* stocks has been shown to be related to the species of vector transmitting the parasite (Janssen and Wijers, 1974). The authors observed that *T. simiae* transmitted by *Glossina brevipalpis* produced an acute disease in pigs but transmission of the same stock of *T. simiae* by *G. pallidipes* caused a chronic disease in the same species of animals (pigs). Likewise, Moloo *et al.* (1992b) compared virulence of a stock of *T. simiae* in pigs transmitted by the two species of *Glossina* and observed that the vector influence on such virulence was less marked than that reported by Janssen and Wijers (1974).

There is no sharp semantic distinction between the terms 'pathogenicity' and 'virulence'. Pathogenicity denotes the ability of an organism to cause disease or the ability

to produce lesions, whereas virulence introduces the concept of degree or the severity of a diseases. For example, virulent organisms show pathogenicity when introduced into the host in very small numbers (Jawetz *et al.*, 1980). Virulence is often measured in terms of numbers of organisms needed to kill a given host when administered by a certain route. It may be expressed as LD_{50} i.e. the number of organisms that must be administered to kill 50% of the animals (Jawetz *et al.*, 1980).

Virulence is also measured by (a) the period to death of the infected host and the time taken for the organism to be detected in an infected host (also referred to as the prepatent period or PPP) (b) the drop in packed red cell volume (PCV) experienced by the infected animal (Murray and Dexter, 1988; Moloo *et al.*, 1992b).

In the past, virulence of trypanosomes was studied in different contexts. Ashcroft (1960) showed that repeated passage of trypanosomes into mice increased their virulence for mice. McNeillage and Herbert (1968) associated virulence with specific variable antigenic types (VATs) located on the surface of trypanosomes. The role of host blood on virulence of *T. congolense* as they undergo cyclical development in the vector was however, not studied. The present investigation was therefore undertaken to determine the influence of host blood fed to the *Glossina* vector on virulence of Savannah and Kilifi sub-types in mice. Studies of biological characteristics of *T. congolense* variants in various species of mammalian hosts are desirable in view of the relevance of such characteristics to trypanosome control.

7.2 MATERIALS AND METHODS

A. Influence of genotype on virulence.

i. Prepatent periods of *Trypanosoma congolense.*

Groups of 50 male *G. m. centralis* were infected with Ng5 and K60/1 *T. congolense* and maintained on rabbits for about a month for trypanosomes to reach the infective stage. Infecting tsetse with *T. congolense* and maintenance of the flies were carried out as described in Chapter 4. Similarly, transmitting trypanosomes from tsetse to mice was done as described in Chapter 6. Briefly, thirty mice, in groups of 10 per genotype, were used in each trial, and the latter was replicated four times. Tail blood was examined daily to determine the day when the mouse became parasitaemic. One hundred fields were examined thoroughly before a mouse was considered uninfected with trypanosomes. The fly used for transmitting trypanosomes into mice will be referred to as the 'fly-transmitter' in this study.

ii. Kinetics of parasitaemia.

Mice were infected with Ng5 and K60/1 through the bite of *G. m. centralis* as described in Chapter 6. Each mouse was examined daily for trypanosome infection. Blood of infected mice was examined on alternate days for 30 days to study the changes in levels of parasitaemia. Trypanosomes were estimated according to the 'matching' method of Herbert and Lumsden (1976).

iii. Survival of infected mice.

Balb/c mice in three groups of 8 animals per cage, were infected with Ng5 and K60/1 as described above in Chapter 7, section 7.2 A. i. and monitored for 45 days to record daily mortalities. The experiment was repeated once. For each *T. congolense*

genotype, the proportion of mice surviving the infection to Day 30 or Day 45 was expressed as a percentage of the total number of mice at the start of the experiment.

B. Influence of host blood on virulence.

i. Prepatent periods of *Trypanosoma congolense.*

Glossina m. centralis in groups of 50 were infected with bloodstream forms of *T. congolense* K60/1 in eland or goat blood in one infective feed as described previously. Other flies were infected with the same trypanosome clone during the first two bloodmeals rather than in one feed. On the 30th day, trypanosomes were transmitted to mice by tsetse bite. Challenged mice were maintained separately in cages corresponding to the two treatments: Treatment 1 = Mice bitten by tsetse infected with eland blood, and Treatment 2 = Mice bitten by tsetse infected with goat blood.

ii. Survival of infected mice.

Mice were infected once only with trypanosomes as described in this Chapter 7 under section 7.2 A. i. They were maintained in separate cages corresponding to three treatments as follows: Treatment 1 = mice bitten by tsetse infected with eland blood. Treatment 2 = mice bitten by tsetse infected with goat blood, and Treatment 3 = mice bitten by tsetse infected with zebra blood. Each treatment was replicated twice. The proportion surviving on Day 30 was expressed as a percentage of the total number of mice at the start of the experiment.

7.3 RESULTS

A. Influence of genotype on virulence.

Kilifi *T. congolense* took a shorter time (13 days) to infect Balb/c mice than the Savannah Ng5 (18 days) ($F = 5.52$, $df = 1$, $P = 0.023$) (Table 24 and Appendix 13).

As shown in Figure 11, Balb/c mice infected with K60/1 and Ng5 revealed parasitaemic peaks on Days 17 and 20 respectively. Furthermore, K60/1 showed more marked fluctuations of parasitaemia than Ng5.

A higher proportion of Balb/c mice infected with Ng5 survived the infection to the 45th day than mice infected with Kilifi *T. congolense* (Fig. 12). Differences in survival rates between the treatments were significant (N = 65, $X^2 = 12.08$, $P = 0.002$).

B. Influence of host blood on virulence.

Figure 13 shows prepatent periods of K60/1 *T. congolense* according to the type of host blood used to infect tsetse. The periods were influenced by the type of host blood used to infect the flies. Trypanosomes transmitted to mice by tsetse infected with goat blood took a mean duration of 11 days to be detected in mice whereas trypanosomes transmitted by flies infected with eland blood took a mean duration of 14 days to be detected (Table 25, Appendix 14). This difference was significant at $P < 0.05$ ($F = 24.12$, $df = 1$, $P = 0.000$). The prepatent period was reduced from 15 to 12 days when the fly transmitters were infected twice with eland blood at the first two bloodmeals, instead of

once. This result was however, not observed with goat blood where the prepatent period was 11 days regardless of the number of infective feeds received by the 'fly-transmitter'.

Figure 14 shows levels of K60/1 *T. congolense* parasitaemia transmitted by *G. m. centralis* infected with goat, zebra and eland blood. Parasitaemias resulting from *G. m. centralis* infected with zebra blood remained relatively low (up to log 7.8) during the first 20 days, and reached the highest level of log 8.4 on the 24th day of infection. In contrast to zebra blood, parasitaemias resulting from tsetse infected with eland and goat blood reached their highest levels of about log 8.4 and log 8.0 respectively around Day 18 i.e. during the early phase of infection.

Host influence on parasite virulence is evident from studies comparing survival of mice according to host blood used to infect tsetse. When the trypanosomes were transmitted to mice by flies infected with zebra blood, none of the infected mice died during the 30-day observation period. However, when trypanosomes were transmitted by tsetse infected with goat blood, 58% of the infected mice died within 30 days and 42% survived. Likewise, trypanosomes transmitted by tsetse flies infected with eland blood killed 33% of the infected mice during the same period (Table 26).
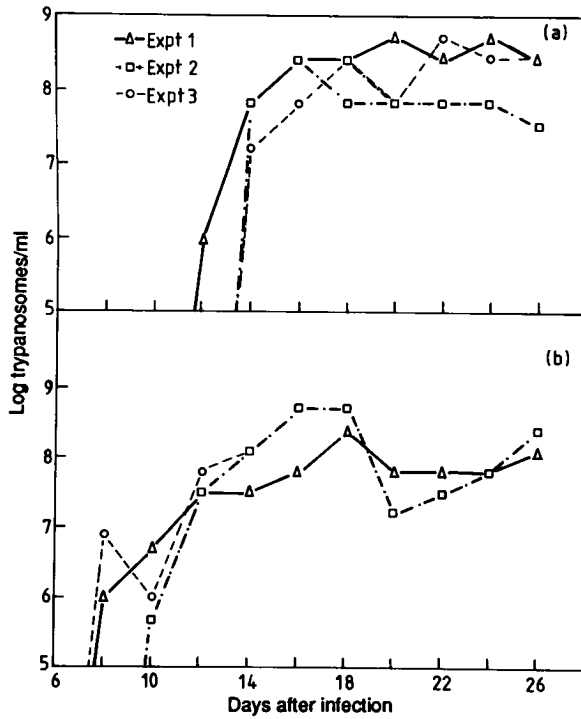
Figure 11 Levels of parasitaemia in Balb/c mice infected with (a) Ng5 and (b) K60/1 by male *G. m. centralis* and monitored for 28 days after infection.

(N = 15 mice for each treatment).
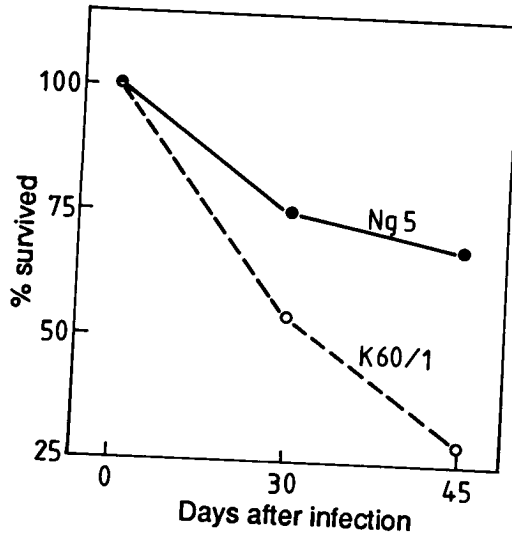
Figure 12 Survival of Balb/c mice infected with Ng5 and K60/1 *T. congolense,* **and monitored for 45 days.**

(Mice were infected singly through the bite of infected *G. m. centralis.* Tsetse were maintained for 30 days after an infective feed on goat blood before being used in transmitting the trypanosomes).
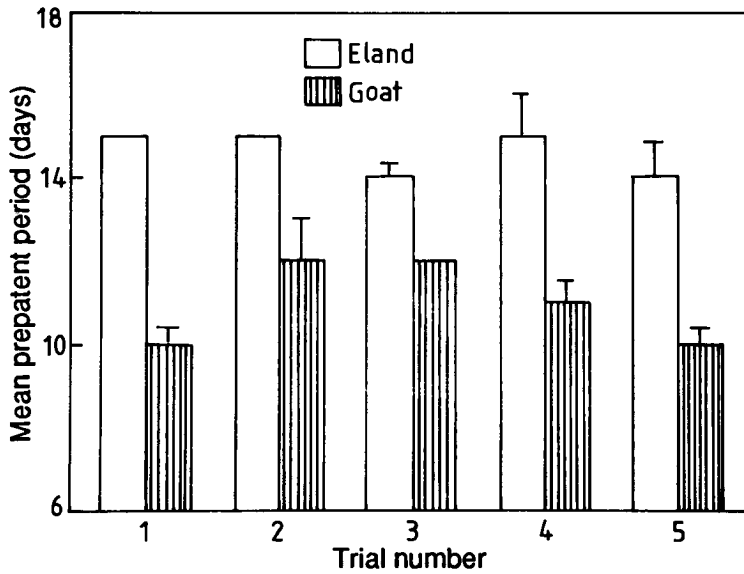
Figure 13 Mean prepatent periods in Balb/c mice of K60/1 *T. congolense* **shown according to the type of host blood used to infect the 'transmitter fly'.**

(Infections in mice were determined by phase contrast microscopy of tail blood at 400x magnification. N = 32 in each treatment).
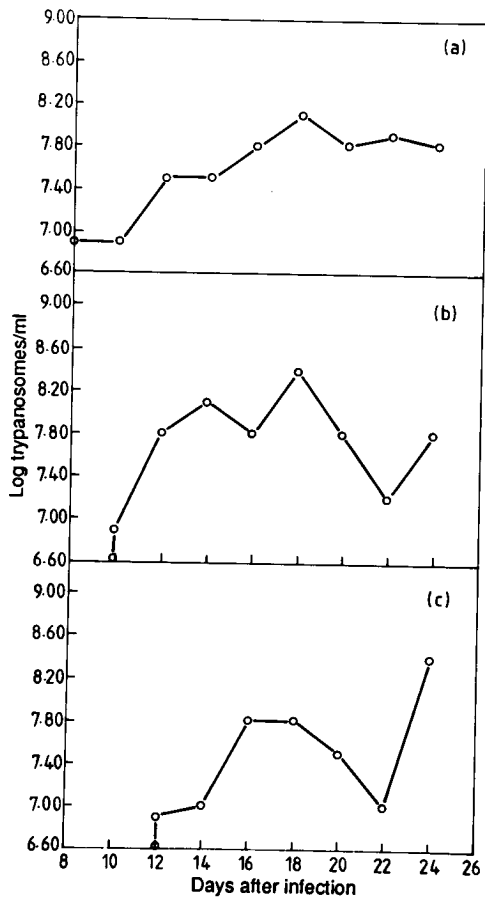
Figure 14 Levels of K60/1 parasitaemia in Balb/c mice according to host blood used to infect *G. m. centralis*: (a) goat, (b) eland and (c) zebra blood.

(Mice were examined for trypanosome infections by microscopy at 400x magnification of tail blood, daily for 28 days. N = 20 in each treatment).

Table 24

Prepatent period and period to death of Balb/c mice infected with Ng5 and K60/1
T. congolense **monitored for 30 days.**

Clone	Prepatent period in days (Mean $\pm$ SE)	N	Days to death (Mean $\pm$ SE)
Ng5	18.00 $\pm$ 1.70 a	40	28.00 $\pm$ 1.70 a
K60/1	13.03 $\pm$ 0.94 b	40	26.00 $\pm$ 4.90 a

Means with different letters between rows are significantly different at *P* < 0.05;

N: Number of mice examined; **SE:** Standard error.

Table 25

Prepatent period (Mean ± SE) of K60/1 in Balb/c mice bitten by *G. m. centralis* **infected with eland and goat blood and pre-infection live body weight (Bwt) in grammes of the mice.**

	Infective blood:	
	Eland	**Goat**
PPP	14.00 ± 0.25 a (n = 33)	11.00 ± 0.45 b (n = 25)
Bwt	19.15 ± 0.97 a (n = 33)	20.00 ± 0.56 a (n = 25)

PPP: Prepatent period between infection and detection of parasitaemia in mice: In parentheses are numbers of mice infected with *T. congolense*. Means with different letters between columns are significantly different at *P* < 0.05; **SE:** Standard error; *F*: Fisher's index; *P*: probability.

Table 26

Proportion of mice surviving in 30 days with K60/1 infection transmitted by male _G. m. centralis_ infected with goat, eland and zebra blood.

Blood	% of surviving mice	% of dead mice
Goat	42.0	58.0
Eland	67.0	33.0
Zebra	100.0	0.0

Mice: N = 16 per treatment, replicated two times.

Fisher's test comparisons: Result

Goat vs. eland: $P = 0.188$ ns

Zebra vs. Goat: $P = 0.005$ s

Zebra vs. Eland: $P = 0.040$ s

s: significantly different at the $P < 0.05$; **ns:** not significant at $P < 0.05$.

7.4 DISCUSSION

Changes in virulence of *T. congolense* following cyclical development in tsetse reported here is probably due to temporary adaptation rather than permanent change in the genome of the trypanosome. According to Masake *et al.* (1988) who studied the chromosome profile of Kilifi *T. congolense* types following transmission by *G. m. centralis* to cattle and goats, the chromosome pattern of this parasite is relatively stable, even after transmission through hosts and tsetse for a period of 12 months. Sacks *et al.* (1980) associated differences in virulence with the capacity of sub-cellular membrane fractions to induce immunosuppression and observed that fractions from acute and chronic strains of *T. brucei* could suppress the production of IgM, and that the level of such suppression correlated well with parasite virulence. Following some observations that trypanosomes subjected to repeated passages in laboratory animals become increasingly monomorphic and virulent, Ashcroft (1960) suggested that virulence was related to the capacity of trypanosomes to differentiate morphologically in hosts. Furthermore, Morrison *et al.* (1978) related virulence to the ability of hosts to limit trypanosome numbers in the blood circulation. Diffley *et al.* (1987) however, suggested that growth rate rather than morphological characteristics was the critical factor governing virulence. The currently observed variations in responses to infections of mice infected with *T. congolense* (Table 25) reported here are probably due to differences in parasite growth rates. This argument is consistent with the observed differences in the prepatent periods of the genotypes.

It is interesting to note that a bloodmeal fed to a tsetse fly at the infective feed can subsequently influence parasite development in a mammalian host. The way host factors change physiological characteristics of a trypanosome in the vector and the stage of development at which this occurs remain unknown. Since it is the metacyclic stage which is finally transmitted to the mammalian host, one can only speculate that the change exerted by host factors on the procyclics persists to the metacyclic stage. An interaction

between the parasite and host factors is probably mediated through membrane receptors on the surface of the trypanosome. Bary (1989) however, demonstrated receptor molecules on trypanosomes but suggested that such molecules were for transport.

This study has highlighted an influence of wildlife and livestock blood on vector forms that can be related to their adaptation to hosts. Wild animals are considered to be true and ancestral hosts of trypanosomes in Africa (Lambrecht, 1980). The reduced virulence of *T. congolense* when exposed to eland blood in the vector could be a reflection of the long evolutionary association and mutual adjustment that may have occurred between this host and the trypanosome. Low virulence ensures perpetuation of parasite species through increased host survival (Lambrecht, 1980). The mutual tolerance between a host and a parasite sometimes fails, however, when a parasite population is introduced into a new area where it is poorly adapted to hosts. This situation has been considered to be a possible cause of sleeping sickness epidemics (Mbulamberi, 1989; WHO, 1993).

The present investigation shows genotype-related differences in virulence for mice. K60/1 *T. congolense* showed a shorter prepatent period, earlier parasitaemic peaks and more marked fluctuations in parasitaemia than Savannah types. This suggests that Kilifi clones used in this study are more virulent to mice than the Savannah types. At the molecular level intra-specific differences have also been reported. It has been found for example, that Kilifi *T. congolense* differed significantly from other *T. congolense* types (Majiwa *et al.*, 1985). Likewise, Knowles *et al.* (1988) showed significant genetic differences between *Nannomonas* stocks from the Kenyan coast.

It is noteworthy that the genetic composition of *T. congolense* from a small area such as the Kenyan coast varies. Knowles *et al.* (1988) postulated that presumptive treatment of cattle with trypanocidal drugs at Kilifi may have preferentially selected *T. congolense* types. Unfortunately, their studies did not elucidate epidemiological factors which led to selection of *T. congolense* variants. Consequently, the genetic variations of

T. congolense isolated from Kilifi, of *T. congolense* originating from Tsavo National Park, Lambwe Valley in Kenya and other parts of Africa remains to be explained. At the Lambwe Valley, natural selection of trypanosomes probably operates together with other mechanisms, to bring about diversity (Mihok *et al.*, 1990). However, Gibson (1990) considered genetic recombination as an important contributor to parasite diversity in the Lambwe Valley. If such is the case, the mechanisms responsible for strain variation at the area are likely to be complex, in view of the large number of possible host-parasite combinations.

The study of parasite variation has identified subgroups of *T. congolense* which differ in virulence. When these observations are applied in the field situations, they may provide clues to questions on factors that maintain trypanosomes at endemic levels or their eruption into epidemic situations (Tait, 1989; Hide and Tait, 1991). If the ability to cause lesions, morbidity and mortality in livestock varies with the genotype, then the control of trypanosomiasis would need to take into account the genetic structure (genotype) of the parasite involved.

CHAPTER 8

GENERAL DISCUSSION

In the past, epidemiological studies on animal trypanosomiasis were hindered by lack of specific, sensitive methods for identifying trypanosomes (Gashumba, 1990; Nantulya, 1990; Hide and Tait, 1991; Majiwa et al., 1993b). Trypanosomes in the subgenus *Nannomonas* were identified on the basis of their lengths and course of infection (Godfrey, 1961; Hoare, 1970, 1972; Stephen, 1986). Unfortunately, trypanosomes in this subgenus vary in morphological features (Gashumba, 1990; Dukes et al., 1991). Also, they may occur as a mixed population of several species (Clarke, 1969; Tarimo et al., 1987; Majiwa and Otieno, 1990). Various factors may further complicate characterisation by behavioural criteria in the laboratory. These include the stage at which the trypanosomes are transmitted from a host, the tsetse species transmitting the parasite and previous exposure to trypanocides (Nantulya et al., 1978; Janssen and Wijers, 1974; Dukes et al., 1991).

Recently, methods have been developed which allow characterisation of trypanosomes to a finer level (Hide and Tait, 1991). These methods have revealed four substantially different genetic variants of *T. congolense*; i.e. Savannah, Kilifi, Tsavo and West African Riverine/Forest strains (Gashumba, 1990; Garside et al., 1994). Several questions have been raised about these variants, including, their response to trypanocidal drugs, their geographical distribution, their variation in virulence and their host specificity (Gashumba, 1990; Hide and Tait, 1991; Tait, 1989; Myler, 1993).

Some researchers have recently addressed these questions in preliminary surveys (Woolhouse et al., 1993; 1994; McNamara et al., 1994). The present study was undertaken to explore the influence of host blood on infection, maturation, transmission and virulence to mice of *T. congolense* DNA variants. It was also conducted to identify an

optimum technique for isolating field *Nannomonas* stocks. Isolates were obtained by transfer of cryopreserved gut-forms into laboratory-reared tsetse and by mouse inoculation. Isolation through procyclic expansion in tsetse was found to be feasible using goat blood. It was achieved in *G. m. centralis,* but was not effective with *G. pallidipes* and *G. m. morsitans.* Inability to isolate trypanosomes in the two other vectors was probably due to lower overall vectorial capacity (Moloo *et al.,* 1992a), and to variation in infectivity of field stocks.

Addition of cholesterol and D(+)-glucosamine increased infection rates of some stocks of *T. congolense* but not all. This further confirmed the large variation in infectivity dynamics of field stocks. It is not known why some trypanosomes do not develop further when inoculated into laboratory rodents (Dukes *et al.,* 1991), culture (Dukes *et al.,* 1989) or when transferred into other vectors (Chapter 4). Dukes *et al.* (1989) attributed failure to isolate *T. b. gambiense* procyclics in *G. m. morsitans* to lack of 'compatibility' between the trypanosome and the vector. These observations indicate that no one method is a panacea to the problem of isolating field stocks. Isolation of trypanosomes by procyclic expansion in tsetse allows characterisation of stocks which do not grow in mice and avoids selection due to long-term sub-passaging in animal hosts. However, procyclic transfer into tsetse often results in very low infection rates (Chapter 4, page 28). The use of chemical compounds such as D(+)-glucosamine enhances trypanosome infection rates but at the expense of fly mortality. The choice of blood products used for cryopreserving procyclics was not a critical factor in this study. Procyclics incubated in different blood mixtures, unheated or heated to inactivate complement, retained parasite viability for a day at room temperature. The procyclic expansion technique has great potential but requires refinement to make it more effective.

The gut of a tsetse fly is a very important site as far as the establishment of *T. congolense* is concerned. Digestive proteases with their potential to kill trypanosomes are secreted there. It is also the site where host antibodies and trypanocidal drugs ingested in a

bloodmeal can come into contact with the trypanosome, resulting in the death of the trypanosome. Studies like the one undertaken here can provide clues as to what factors contribute to parasite survival in the vector. This information might improve our knowledge of the epidemiology of trypanosomiasis, and therefore provide new tools for effective disease control. A species-specific host blood influence on *T. congolense* midgut establishment in *G. m. centralis* was demonstrated (Chapter 5, page 55). Goat and zebra blood supported higher midgut infections of *T. congolense* than blood from other host species. The mechanism by which this occurs is unknown. Perhaps zebra red blood cells possess 'exposed' carbohydrate moieties that bind to lectins, thereby enhancing trypanosome survival. However, this explanation raises many questions. For example, why would zebra and goat RBCs be more effective in blocking lectins than those of other host bloods? It is possible that the carbohydrate residues on the surface of RBCs of zebra blood fit tsetse lectin better or have more residues exposed than other RBCs. The factor that supports *T. congolense* infection rates in host blood must resist enzyme digestion to be of value. For this reason lipids appear to be logical candidates (Mihok *et al.*, 1993). It is curious that two non-preferred hosts, goats classified in a different family (Bovidae) from zebras (Equidae) (Kingdon, 1974) support similar high trypanosome infection rates. Isolation and characterisation of the host factor(s) responsible is now necessary to resolve the causes of these odd patterns. From a practical viewpoint, this study suggests that areas with many goats should have higher rates of trypanosome infections in tsetse. Zebra being a less-favoured host of *Glossina* (Weitz, 1963) probably has a lesser impact on infection rates. Furthermore, the results with zebra blood serve to highlight: (a) that some vital epidemiological factors on trypanosomiasis in wildlife remain unknown and (b) the need for more studies with wildlife species.

Maudlin *et al.* (1984) suggested that host serum factors are responsible for preventing *T. congolense* midgut infections becoming established in *G. m. morsitans*. Mihok *et al.* (1993) demonstrated that increasing the packed red cell volume (PCV) of a

bloodmeal increased trypanosome infection rates in *G. m. morsitans*. Subsequently, Mihok *et al.* (1995) noted differences between tsetse species in susceptibility to *T. congolense* infections when infected with different reconstituted blood diets. Serum meals increased midgut infections of a Savannah-type *T. congolense* in *G. m. centralis* whereas red blood cells increased *T. congolense* infections in *G. m. morsitans*. Ingram and Molyneux (1988) suggested that human ABO(H) red blood cells possess surface carbohydrate moieties that bind specifically to lectins in tsetse tissues. Lectins were implicated in killing trypanosomes (Maudlin and Welburn, 1987; 1988; Welburn *et al.*, 1989). Lectins and proteases are stimulated by a bloodmeal (Welburn *et al.*, 1989; Stiles *et al.*, 1990; Osir *et al.*, 1993). It can be concluded that the type of host blood and the quality and the packed red cell volume (Mihok *et al.*, 1995) should be taken into consideration when designing experiments on vectorial capacity.

Infection rates in *G. m. centralis* were generally high on Day 3 but considerably lower between Days 5 and 10 (Chapter 5, pages 55-56). This result was true regardless of the genotype. Days 5 to 10 may be the period when specific immune-related molecules, trypanocidal or regulatory act. Titres of midgut lectins in *G. m. morsitans* peak around Day 6 after a bloodmeal. Various defence molecules exist in insect vectors. Some of these may be non-specific, acting as immune 'surveillance' (Ingram and Molyneux, 1991) whereas others play more specific roles. For example, defence molecules against filarial parasites in the vectors of filaria include inducible proteases and haemolymph lectins (Ingram and Molyneux, 1991; Ham *et al.*, 1991; Ham *et al.*, 1994). Also, the foregut of some mosquitoes is characterised by sclerotised teeth and spines occurring in groups or rows called armatures. In *Anopheles gambiae s.l.*, armatures have been reported to damage microfilaria (Bryan and Southgate, 1988). Inspite of these defences, many parasites succeed in completing development to mature stages.

The present study revealed host-specific influence on maturation of *T. congolense* (Chapter 6, page 79). The observation that *Glossina m. centralis* infected with zebra

blood consistently supported maturation of higher proportions of *T. congolense* midgut infections than tsetse infected with blood of other host species suggests presence in zebra blood, of factors essential for completing cyclical development. This is consistent with earlier suggestions that serum factors, probably lipids, promote maturation of trypanosomes (Maudlin *et al.*, 1984). However, the actual trigger for maturation has not been established and further research on this aspect is desirable.

Lectin involvement in parasite maturation in insects has been suggested in other studies. For example Welburn and Maudlin (1990) blocked maturation of *T. congolense* and *T. b. brucei* in *G. m. morsitans* by adding melibiose, the haemolymph lectin, to the bloodmeal. Perrone *et al.* (1986) found differences in lectin binding patterns on the salivary glands of *Aedes aegypti*. Therefore, we cannot rule out the possibility that lectins were involved in maturation of *T. congolense* in *G. m. centralis* fed on different animal species in the current study.

This present study further showed sex-related differences in maturation and transmission rates of *T. congolense* (Chapter 6, pages 79-80). In general, male tsetse facilitates maturation significantly more midgut trypanosomes and infect a higher proportion of mice than females. This observation is in close consistence with earlier observations by Distelmans *et al.* (1982) who reported higher *T. congolense* maturation rates in male than in female *G. p. palpalis*, Moloo *et al.* (1992a) who reported similar findings with *T. congolense* infection rates in male than female *G. pallidipes*, and Moloo *et al.* (1994b) who found similar results with *T. simiae* in several *Glossina* species. Contrary to these findings however, Maudlin *et al.* (1991) analysed data from several sources and concluded that maturation of *T. congolense* in tsetse was independent of fly sex. These discrepancies in maturation rates may be a reflection of intrinsic differences in the physiology of the two sexes. The higher transmission rates of male versus female tsetse (Chapter 6, page 79) have important implications in tsetse control using the Sterile Male Technique (SMT). This method which relies on releasing a large number of sterilised

males into the field to mate with fertile females results in non-viable embryos that cannot develop to adults (Molyneux *et al.*, 1982; Dame, 1970; FAO, 1992). However, tsetse sterilised with radiation or chemosterilants retain the ability to transmit trypanosomes (Moloo, 1982). Release of such insects into areas where the disease already exists may therefore increase the rate of trypanosomiasis transmission and worsen the disease situation instead of improving it, especially if the sterilised males fly survive long enough for trypanosomes to mature. In view of the higher transmission rates of *T. congolense* by male than female *G. m. centralis* revealed in this investigation, the need to devise ways of reducing the vectorial capacity of sterilised tsetse before they are released in the field is desirable so as to make the SMT more effective in reducing trypanosome transmission. However, such devices should not alter the ability of these insects to compete with other males for mating females.

The observation in this study that the influence of host blood on metacyclic development of *T. congolense* was host specific can be interpreted to suggest that various animal hosts possess some factors which affect the growth rates and parasitaemia patterns of *T. congolense* when tsetse flies are fed on animals infected with this parasite. The fact that transmission rates to hosts were similar regardless of the type of host blood used to infect the transmitting flies probably suggests that such factors have no effect on the vectorial capacity of tsetse or infectivity rates of *T. congolense*. However, *Trypanosoma congolense* DNA types showed variation in virulence to mice. Therefore any plans for control of animal trypanosomiasis ought to take into account the DNA subtypes of the trypanosomes involved in an area, since differences in morbidity, mortality and responses to trypanocidal drugs are shown to vary according to trypanosome types.

Further studies which should concentrate on two priority areas are recommended. These are (i) determining the factor(s) in wildlife and livestock blood responsible for supporting establishment and maturation of *T. congolense* and (ii) assessing infectivity, responses to trypanocides and virulence in the natural hosts of *T. congolense* DNA

variants. The knowledge gained from these studies can have a positive impact on trypanosomiasis control.

SUMMARY

1. An investigation using *Glossina m. centralis* was undertaken to determine the influence of host blood on infection, maturation, transmission rates and virulence to mice of *T. congolense* DNA types. The study was also conducted to identify an optimum technique for isolating *Nannomonas* stocks from the field.

2. Isolation of *T. congolense* through procyclic expansion in tsetse was most effective in *G. m. centralis,* and was not effective in *G. pallidipes* and *G. m. morsitans*. This variation was attributable to probable differences in the trypanocidal factors found in the gut environment of these species.

3. Cholesterol and D(+)-glucosamine increased infection rates of *Nannomonas* procyclics in *G. m centralis*. However, glucosamine also caused fly mortality.

4. The choice of blood products used for cryopreserving procyclics was not a critical factor. Procyclics incubated in different blood mixtures, heated to inactivate complement or unheated, retained parasite viability for a day at room temperature.

5. Isolation of trypanosomes by mouse inoculation was more effective in Balb/c than Swiss mice.

6. Goat and zebra blood supported higher midgut *T. congolense* infections than pig, donkey, black rhinoceros, Thomson's gazelle and eland blood. Differences in specificity of host red blood cell carbohydrate moieties for tsetse gut lectins and other host factors in sera might be responsible for the different capabilities to support mid gut infections with *T. congolense*.

7. The pattern of *T. congolense* Savannah, Kilifi and Riverine infection in *G. m. centralis* infected with goat, black rhino or cow blood was similar during the first 10 days of infection. Rates were highest (up to about 90%) on Day 3, dropped between days 5 and 9 to reach constant values (ranging from 5% to 45%) by Day 10. Day 10 midgut infection rates did not differ significantly from those on Day 21.

8. At Day 10, midgut infection rates of Savannah *T. congolense* were higher than those of Kilifi and Riverine *T. congolense* in *G. m. centralis* infected with goat blood. However, no genotype-related differences in maturation and transmission rates were found.

9. Goat and zebra blood supported higher labral infection rates with *T. congolense* than pig, donkey, black rhinoceros, Thomson's gazelle and eland blood. Zebra blood supported the highest metacyclic loads in the hypopharynx compared to blood of other host species. This observation is probably attributable to differences in levels of stimulation of the 'maturation lectins' in tsetse flies.

10. Male *G. m. centralis* matured more midgut infections than females only when infected with eland and zebra blood. A higher proportion of mice became infected with *T. congolense* when bitten by a male fly than when bitten by a female fly.

11. Infection rates of Savannah *T. congolense* in mice were similar to those of Kilifi *T. congolense*, when infection was initiated by the bite of *G. m. centralis*. Transmission rates of *T. congolense* DNA types to mice were not influenced by host blood used to infect the transmitting fly.

12. Kilifi *T. congolense* took a shorter time to infect and kill Balb/c mice and showed earlier parasitaemic peaks than the Savannah type, suggesting that the former genotype was more virulent.

13. *Trypanosoma congolense* was detected in mice earlier when the transmitting tsetse was infected with goat blood than when the transmitting tsetse was infected with eland blood, suggesting host effects on adaptation of metacyclics during the early phase of development in a mammal.

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LIST OF ABBREVIATIONS

Chol	Cholesterol
°C	Degree centigrade (or Celcius)
$	US dollar
FCS	Foetal Calf Serum
G	Gauge
GlcN	D(+)-glucosamine
GlcNAc	N-acetyl-D-glucosamine
h	hour
ICIPE	International Centre of Insect Physiology and Ecology
ILRAD	International Laboratory for Research on Animal Diseases
km	kilometre
m	metre
mg	milligram
RLOs	Rickettsia-like Organisms
TDRC	Tropical Diseases Research Centre
µl	microlitre
WHO	World Health Organization

SCIENTIFIC NAMES OF ANIMALS

Common Name..**Scientific Name**

Common Name	Scientific Name
Eland	*Tragelaphus (Taurotragus) oryx* (P.)
Giraffe	*Giraffa camelopardalis* (L.)
Zebra	*Equus burchelli* (G.)
Thomson's gazelle	*Gazella thomsoni* (G.)
Rhinoceros (black)	*Diceros bicornis* (L.)
Goat	*Capra hircus* (L.)
Cattle (Boran)	*Bos indicus* (L.)
Domestic pig	*Sus scrofa* (L.)
Donkey	*Equus asinus* (L.)

Adapted from Haltenorth. and Diller (1980) and Kingdon (1974).

APPENDICES

Appendix 1

Trypanosome infection rates in cattle determined by microscopy of buffy coat at 400x and antigen detection (latex agglutination) test during trial 3 at Lambwe Valley (March, 1994).

Animal	PCV(%)	Microscopy			Latex agglutination		
		Tv	Tb	Tc	Tv	Tb	Tc
5	31	-	-	-	-	W	-
6	35	-	-	-	-	W	-
7	33	-	-	-	W	W	-
8	30	-	-	-	S	S	M
11	35	-	-	-	-	-	-
15	32	-	-	-	-	-	-
16	29	-	-	-	-	-	-
17	32	-	-	-	-	-	-
18	26	-	-	-	S	W	-
19	32	-	-	-	-	-	-
21	33	-	-	-	W	W	W
23	28	-	-	-	-	-	-
24	34	-	-	-	-	-	-
26	30	-	-	-	-	W	W
28	30	-	-	-	-	-	-
29	26	-	-	-	-	W	W
30	27	-	-	-	W	W	W
32	28	-	-	-	-	W	W
37	28	-	-	-	-	-	-
38	33	-	-	-	W	S	W
40	34	-	-	-	S	S	S
43	27	-	-	-	W	W	W
44	35	-	-	-	M	M	M
49	29	-	-	-	S	M	-
51	31	-	-	-	W	S	S
52	24	-	-	-	-	-	-
55	31	-	-	-	W	-	-
56	32	-	-	-	-	-	-
59	33	-	-	-	M	M	M
60	32	-	-	-	S	S	-
					-	-	-

+: Infected with trypanosomes; **Tv:** *T. vivax*; **Tc:** *T. congolense*; **Tb:** *T. brucei*;

S: Strong; **W:** Weak; **M:** Medium; **PCV:** Packed red cell volume.

Appendix 2

Trypanosome infections in cattle determined by microscopy of buffy coat at 400x and antigen detection (latex agglutination) test of plasma during trial 3 at Rusinga Island (March, 1994).

Animal	PCV (%)	Microscopy			Latex agglutination		
		Tv	Tb	Tc	Tv	Tb	Tc
1	27	-	-	-	-	-	-
2	32	-	-	+	S	S	S
3	35	-	-	-	S	S	S
4	28	-	-	-	S	-	M
5	26	-	-	-	-	-	-
6	31	-	-	-	-	-	-
7	33	-	-	-	-	-	-
8	34	-	-	-	W	W	W
9	32	-	-	-	S	S	S
10	32	-	-	-	W	S	W
11	30	-	-	-	S	-	-
12	30	-	-	-	W	W	-
13	24	-	-	-	-	-	-
14	23	-	-	-	S	S	S
15	27	-	-	-	-	S	S
16	31	-	-	-	W	-	W
17	27	-	-	-	-	-	-
18	35	+	-	+	W	W	W
19	30	-	-	+	W	W	W
20	35	-	-	-	-	-	-
21	24	-	-	-	W	W	W
22	22	-	-	-	-	W	-
23	27	-	-	+	S	M	M
24	28	-	-	-	M	-	M
25	29	-	-	-	-	-	-
26	32	-	-	-	-	-	-

+: Infected with trypanosomes; **Tv:** *T. vivax*; **Tc:** *T. congolense*; **Tb:** *T. brucei*;

S: Strong; **W: Weak;** **M**: Medium; **PCV:** Packed red cell volume.

Apendix 3

Patterns of *T. congolense* (K60/1) midgut infections in *G. m. centralis* membrane fed on double goat or double eland or eland followed by goat or goat followed by eland blood. Tsetse were dissected 10 days after infection: Analysis of variance.

Source	DF	SS	MSS	F	P
Model	7	822.76	117.54	4.97	0.034
Error	6	141.90	23.65		
Corrected Total	13	964.67			

DF: Degrees of freedom;   **SS**: Sum of squares;   **MSS**: Mean sum of squares;
F: Fisher's index;   **P**: Probability.

Appendix 4

**Effect of N-acetyl-D-glucosamine (GlcNAc) and Cholesterol (Chol) on midgut
infection rates (%) of** *T. congolense* **Ng5 in** *G. m. centralis* **fed on goat blood and
dissected 10 days later : Analysis of variance.**

Source	DF	SS	MSS	F	P
Model	1	2590.87	2590.87	12.65	0.006
Error	9	1842.96	204.77		
Corrected Total	10	4433.84			

DF: Degrees of freedom;   **SS**: Sum of squares;   **MSS**: Mean sum of squares;
F: Fisher's index;   **P**: Probability.

Appendix 5

Effect of N-acetyl-D-glucosamine (GlcNAc) and cholesterol (Chol) on midgut infection rates(%) of *T. congolense* **K60/1 in** *G. m. centralis* **fed on goat blood and dissected 10 days later: Analysis of variance.**

Source	*DF*	SS	MSS	*F*	*P*
Model	1	4056.54	4056.54	16.60	0.001
Error	14	3422.08	244.43		
Corrected Total	15	7478.62			

DF: Degrees of freedom;   **SS**: Sum of squares;   **MSS**: Mean sum of squares;   *F*: Fisher's index;   *P*: Probability.

Appendix 6

Influence of host blood on labrum infection rates of *T. congolense* **in** *G. m. centralis*:
Analysis of variance.

Source	DF	SS	MSS	F	P
Blood	5	1259.34	251.87	10.09	0.000
Genotype	1	45.84	45.84	1.84	0.19
Sex	1	85.77	85.77	3.43	0.077
Blood x sex	4	89.80	22.45	0.90	0.48
Genotype x sex	1	6.43	6.42	0.26	0.62

DF: Degrees of freedom; **SS:** Sum of squares; **MSS:** Mean sum of squares;
F: Fisher's index; *P*: Probability.

Appendix 7

Influence of host blood on hypopharynx infection rates of *T. congolense*[*] **in**
G. m. centralis :**Analysis of variance.**

Source	DF	SS	MSS	F	P
Blood	5	1013.19	202.64	7.75	0.000
Clone	1	78.53	78.52	3.00	0.097
Sex	1	187.98	187.98	7.19	0.014
Genotype x sex	1	28.10	28.10	1.08	0.31
Blood x sex	4	77.48	19.37	0.74	0.57

DF: Degrees of freedom;   **SS**: Sum of squares;   **MSS**: Mean sum of squares;

F: Fisher's index;   *P*: Probability;   *T. congolense*[*] = NG5 and K60/1.

Appendix 8

Influence of host blood on development of 'heavy' metacyclic infections in the hypopharynx of *G. m. centralis* **by genotype: Analysis of variance.**

Source	DF	SS	MSS	F	P
Blood	5	713.28	142.66	4.160.	0.016
Genotype	1	42.06	42.06	1.23	0.29
Blood x Genotype	3	45.91	15.30	0.45	0.72

DF: Degrees of freedom;   **SS:** Sum of squares;   **MSS:** Mean sum of squares;

F: Fisher's index;   *P*: Probability.

Appendix 9

Influence of host blood (zebra, pig and donkey) fed to tsetse during the first bloodmeal on transmission rates of *T. congolense* **to mice by** *G. m. centralis*. **Analysis of variance.**

Source	*DF*	SS	MSS	*F*	*P*
Model	2	297.99	149.090	2.10	0.27
Error	3	212.99	71.00		
Corrected Total	5	510.98			

DF: Degrees of freedom; SS: Sum of squares; MSS: Mean sum of squares; *F*: Fisher's index; *P*: Probability.

Appendix 10

Influence of host blood (goat and eland) fed to tsetse during the first bloodmeal on transmission rates of *T. congolense* **to mice by** *G. m. centralis*:**. Analysis of variance.**

Source	DF	SS	MSS	F	P
Model	1	610.59	610.59	1.06	0.34
Error	7	4029.74	575.68		
Corrected Total	8	4640.33			

DF: Degrees of freedom;   **SS:** Sum of squares;   **MSS**: Mean sum of squares;
F: Fisher's index;   *P*: Probability.

Appendix 11

Transmission rates of $T.$ *congolense* **into Balb/c mice by** $G.$ *m. centralis* **by genotype of** $T.$ *congolense*: **Analysis of variance.**

Source	DF	SS	MSS	F	P
Model	2	458.47	229.50	0.35	0.71
Error	14	7182.48	653.00		
Corrected Total	16	7641.42			

DF: Degrees of freedom;   **SS:** Sum of squares;   **MSS**: Mean sum of squares;
F: Fisher's index;   *P*: Probability.

Appendix 12

Transmission rates of *T. congolense* **into Balb/c mice by fly sex: Analysis of variance.**

Source	DF	SS	MSS	F	P
Blood	2	99.59	49.80	0.35	0.71
Clone	2	11.42	5.71	0.04	0.96
Sex	1	757.48	757.48	5.31	0.037
Genotype x sex	1	89.92	89.92	0.63	0.44

DF: Degrees of freedom;   **SS**: Sum of squares;   **MSS**: Mean sum of squares;
F: Fisher's index;   *P*: Probability.

Appendix 13

Prepatent period (Mean ± SE) of K60/1 *T. congolense* **in Balb/c mice monitored for 30 days: Analysis of variance.**

Source	*DF*	SS	MSS	*F*	*P*
Model	1	314.03	314.03	5.52	0.023
Error	42	2387.86	56.85		
Corrected Total	43	2701.89			

DF: Degrees of freedom;   **SS:** Sum of squares;   **MSS:** Mean sum of squares;

F: Fisher's index;   *P*: Probability.

Appendix 14

Prepatent periods (Mean ± SE) of K60/1 *T. congolense* **in Balb/c mice bitten by** *G. m. centralis* **infected with goat or eland blood. N = 58: Analysis of variance.**

Source	DF	SS	MSS	F	P
Model	1	81.77	81.77	24.12	0.000
Error	56	189.82	3.39		
Corrected Total	57	271.59			

DF: Degree of freedom; **SS:** Sum of squares; **MSS:** Mean sum of squares;

F: Fisher's index; *P*: Probability.